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Monocyte Secretory Responses : Relationship to Periodontitis

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MONOCYTE SECRETORY RESPONSES: RELATIONSHIP TO
PERIODONTITIS

Scott Warren Garrison

B. S., Ursinus College, 1984

D. D. S., Case Western Reserve University, 1984

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Submitted in Partial Fulfillment of the

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Master of Dental Science

at

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Master of Dental Science Thesis

MONOCYTE SECRETORY RESPONSES: RELATIONSHIP TO
PERIODONTITIS

Presented by

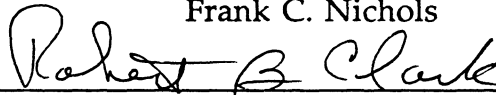
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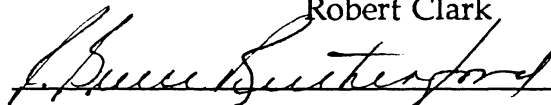
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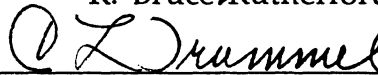
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INTRODUCTION

The pathogenesis of adult periodontitis involves a complex interaction between bacterial factors and the host immune response. Although a great deal has been learned about this interaction, a poor understanding of the susceptibility of individuals to periodontal disease processes still exists. Clinical research has attempted to correlate periodontal attachment loss with specific alterations in gingival crevicular fluid (8,43,44), subgingival bacterial flora (12) or histological surveys (45,48,60). This approach has generated useful information, however, results have generally not provided satisfactory methods for predicting disease activity or patient susceptibility. Hundreds of bacterial species have been identified in subgingival plaque (39). The extreme complexity of this subgingival ecology prevents the identification of one or more bacteria as the causative factor in periodontitis. Furthermore, the importance of bacterial products such as collagenase, leukotoxins, and lipopolysaccharide (LPS) are hard to assess *in situ*. The examination of crevicular fluid for levels of soluble immune mediators such as prostaglandins, interleukins, complement fragments, and/or cellular enzymes, fails to identify the role of these mediators in promoting pathogenic processes within established periodontitis lesions. Furthermore, bacteria present in the crevice or pocket release a number of proteolytic enzymes which may alter those mediators being examined (68). Humoral immune responses to suspected periodontal pathogens have established the existence of a systemic immune response to oral microorganisms associated with periodontitis. However, these responses have not provided information useful in predicting periodontal breakdown or patient susceptibility to periodontitis.

With the development of counterflow centrifugation it is now possible to rapidly isolate, in high purity, monocytes from fresh peripheral blood. By combining this cell isolation technique with *in vitro* cell culture methods, a different approach to the study of immune function and regulation in periodontitis is possible. In addition, this approach is conveniently applied to assessment of monocyte responses and their relationship to periodontitis in dental patients. Therefore, these methods were employed in the investigation of immune function as described below.

This thesis proposes to study whether cells from human subjects possess an inherent ability to respond to LPS which can be correlated with the degree of periodontal destruction within the same individuals. According to this model, LPS released from the subgingival flora activates cells of the monocyte and lymphocyte lineages to various levels of immune and inflammatory function. Alterations in this process could occur through the innate variability of LPS-responsiveness. One aspect of LPS-induced immune and inflammatory responses involves the release of soluble mediators, such as, IL-1 and PGE₂ from monocytes. Products such as these, play an important role in the regulation of inflammatory and immune processes. Therefore, alterations in the levels of these, and other regulating substances could have profound effects on the character of the local immune and inflammatory responses. Alterations in the LPS-stimulated release of these substances can also occur through the activities of natural immune regulatory substances such as gamma interferon (IFN- γ). Prior to conducting a clinical investigation of this hypothesis, it was necessary to partially characterize LPS and IFN- γ effects on monocyte release of PGE₂ and IL-1. Therefore, the three primary goals of this research project were to : 1) characterize and compare the relative potencies of LPS preparations from various suspected periodontal pathogens

in the stimulation of PGE₂ and/or IL-1 release 2) analyze the effects of IFN- γ on LPS stimulated responses ,and lastly, using this information, 3) conduct a pilot clinical study of the LPS-responsiveness of patients and its correlation to periodontitis-susceptibility.

An abundance of evidence suggests the importance of bacterial lipopolysaccharide (LPS) in the pathogenesis of periodontitis. LPS is a constituent of the cell wall of gram negative bacteria and is released upon cell lysis or through a process similar to yeast "budding off" (9). Because gram negative bacteria predominate within the sulcus and periodontal pocket in periodontitis (32), LPS may participate, to a major degree, in periodontal pathogenic mechanisms. Research has shown that ³H-LPS can penetrate sulcular epithelium (59), thus demonstrating the ability of LPS to penetrate clinically healthy, gingival soft tissues. In addition, application of LPS to tooth surfaces is associated with vascular leakage in the adjacent connective tissue (51). The positive correlation between levels of LPS in the gingival fluid with clinical (60) and histological (61) signs of periodontal inflammation gives additional support for the importance of LPS in periodontitis.

Research conducted with C3H murine strains has determined that the ability of murine cells to respond to LPS is genetically determined (66) and that the gene locus is on chromosome 4 (75). The possibility that LPS responses may be genetically determined in humans raises important considerations with respect to diseases, such as periodontitis, where host cells are exposed to bacterial LPS. LPS-induced pathologic changes within the periodontium would likely involve both lymphocytes and monocytes.

Various findings support the role of cells from the monocyte-macrophage lineage in periodontal pathogenesis. They are found in elevated numbers in diseased periodontal connective tissues (58). More importantly,

monocytes release many substances capable of modulating the function of surrounding cells and connective tissue metabolism. Specifically, monocytes exposed to LPS, release PGE₂ (29) and IL-1 (2). The importance of PGE₂ in periodontitis is suggested by the increased amounts found in periodontally diseased tissues (18,34,38) and crevicular fluid (44). The proinflammatory, immunosuppressive, and bone resorbing activities of PGE₂ are undoubtedly important in the regulation of periodontal destruction. IL-1 also possesses bone resorbing activity (19) and has been found in crevicular fluid (8). However, the effects of IL-1 on other cells are potentially more important with respect to periodontal destruction. IL-1 stimulates the release of PGE₂ (11) and collagenase (50) from fibroblasts. In this manner, LPS stimulation results in PGE₂ and collagenase release directly from monocytes and indirectly from fibroblasts, via IL-1. Recently, the release of IL-1 and PGE₂ have been shown to be interrelated (28). It has been demonstrated that IL-1 alone, stimulated PGE₂ release, whereas, exogenous PGE₂ inhibited IL-1 release. Furthermore, inhibition of PGE₂ release by indomethacin resulted in elevated IL-1 release. Another important function of IL-1 pertains to its role as a cofactor in the activation of T lymphocytes. Treatment of T cells with IL-1 results in expression of interleukin-2 (IL-2) receptors (25). This allows T cell activation by IL-2, resulting in blastogenesis and the release of IFN- γ (30).

Recent evidence suggests that in certain stages of chronic periodontitis, T lymphocyte numbers predominate over B cells within periodontal connective tissues (45,60). Interestingly, it has also been demonstrated that LPS-treated monocytes can stimulate the release of IFN- γ from T lymphocytes (30). This finding has important implications for the study of LPS-monocyte interactions in periodontal pathogenesis. IFN- γ effects various aspects of monocyte function, including elevated expression of Ia antigen (27) and Fc

receptors (20,49), resulting in increased efficiency of phagocytosis and antigen presentation. Furthermore, LPS stimulated monocyte responses such as tumoricidal activity and IL-1 release are increased with IFN- γ treatment (2,47). Because T cells and monocytes are both elevated within inflamed periodontal tissues, examination of the effect of IFN- γ on LPS-stimulated monocyte responses was also include in this work.

CHAPTER 1

Lipopolysaccharide stimulated PGE₂ release from human monocytes:
comparison of lipopolysaccharides prepared from suspected periodontal
pathogens.

ABSTRACT

Lipopolysaccharides (LPS) prepared from the suspected periodontal pathogens *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis*, *B. intermedius* and *Wolinella recta* were compared to *Salmonella typhimurium* LPS, for their capacity to stimulate PGE₂ release from human monocytes. Counterflow isolated monocytes were cultured with control medium or media containing 10 ug/ml LPS. Media were then exchanged every 24 hours for a total of 72 hours. *Salmonella* and *Wolinella* LPS preparations demonstrated 7-fold greater PGE₂ release than *B. gingivalis* and 5-fold greater than *A. a.* and *B. intermedius*. PGE₂ release was found to decrease over time with all LPS preparations except *Wolinella*. The potency of the LPS preparations is tentatively ranked as follows: *Wolinella* \geq *Salmonella* $>$ *A. a.* $>$ *B. intermedius* \geq *B. gingivalis*. These findings demonstrate that LPS preparations from suspected periodontal pathogens are capable of stimulating PGE₂ release from human monocytes. The high potency and prolonged stimulation of PGE₂ release with *Wolinella* LPS suggests unusual toxic properties which may exert a greater influence in the pathogenesis of destructive periodontal diseases.

INTRODUCTION

According to current evidence, the progression of adult periodontitis may be associated with the presence of certain gram negative bacteria, including *Wolinella recta*, *Actinobacillus actinomycetemcomitans* (*A. a.*), and *Bacteroides* species (12). *A. a.* has also been associated with the progression of localized juvenile periodontitis (63). Although an association with clinical attachment loss may implicate these organisms in the pathogenesis of periodontal diseases, the specific mechanisms by which they promote peri-

odontal destruction remain to be established. Each of the previously described gram negative species possess lipopolysaccharide (LPS) as a cell wall constituent. However, numerous structural and functional differences have been reported for lipopolysaccharides isolated from these suspected periodontal pathogens (4,24,26,36,41,69). Although the release of LPS from organisms within the subgingival plaque has not been related to periodontal destructive processes, LPS levels in gingival crevicular fluid have been positively correlated with clinical and histologic signs of gingival inflammation (61,62). In addition, isotopically-labelled LPS can penetrate intact sulcular epithelium (59) and the topical application of LPS to tooth surfaces leads to increased vascular permeability within the adjacent gingiva (51). Other pathological responses elicited by LPS include abscess formation, osteoclast-mediated bone resorption, pyrogenicity, and initiation of the localized Schwartzman reaction (reviewed in 16). These responses have previously been used to compare the relative potencies of LPS preparations, however, the capacity to elicit these pathologic responses may not correlate with periodontal tissue destruction. Furthermore, LPS potencies within these biological assay systems may not be relevant to the pathogenesis of periodontal diseases. The purpose of the present study was to assess the relative potencies of these LPS preparations within the context of a specific cellular response considered important in the pathogenesis of periodontitis.

Elevated release of prostaglandin E_2 (PGE_2) is a prominent response of monocytes when exposed to LPS (55). In addition, release of PGE_2 has been proposed as a mechanism involved in the pathogenesis of periodontitis (18,38). The ability of PGE_2 to modulate inflammatory and immune reactions, as well as bone resorption support this idea (65). Furthermore, PGE_2 (18,34,38) and mononuclear phagocytes (58) are increased in the connective tissue of

adult periodontitis lesions. Although an impressive release of PGE₂ can be demonstrated following treatment of monocytes with LPS from *Salmonella typhimurium* ; there is little information available concerning PGE₂ release elicited by LPS preparations isolated from suspected periodontal pathogens. In the present study, we examined the capacity of LPS preparations isolated from *Bacteroides gingivalis*, *B. intermedius*, *Actinobacillus actinomycetemcomitans*, and *Wolinella recta* to elicit elevated PGE₂ release from human monocytes. LPS from *Salmonella typhimurium* was used as a reference for comparative purposes, since considerable data exists concerning its structure and biologic activities.

METHODS AND MATERIALS

Cell Separation : Monocytes were separated from citrated whole venous blood of random human donors by ficoll-Hypaque sedimentation (400 x g x 35 min.) at 10°C. The mononuclear leukocyte rich fractions were collected, diluted, and washed two times in Ca⁺⁺ and Mg⁺⁺ free, phosphate buffered saline (PBS) (125 x g x 15 min.) at 10°C. The cells were then counted electronically (Coulter Electronics) and monocyte enrichment achieved by counterflow centrifugation (Beckman Instruments). Autologous plasma (1%) was added and monocytes sedimented (125 x g x 15 min.) and resuspended in MCDB 104 medium (37) supplemented with 1% defined heat inactivated fetal calf serum (Hyclone), 100 U/ml penicillin and 100 mcg/ml streptomycin. Cells were counted and plated in plastic (Falcon Plastics) 16mm wells (1 x 10⁶ cells/ml per well). At 2 hours the cells were washed two times in medium to remove any non-adherent cells. Cultures were incubated at 37°C in an H₂O saturated 5% CO₂ atmosphere. Cell viability was routinely confirmed by trypan blue exclusion. Treatments consisted of the following: LPS (10 ug/ml)

or control medium. Media were exchanged at 24, 48, and 72 hours and the supernatants assayed for PGE₂ content.

LPS Preparations: LPS was obtained from the following bacterial strains: *Salmonella typhimurium* (Difco Laboratories), *Bacteroides gingivalis*, *Bacteroides intermedius*, *Actinobacillus actinomycetemcomitans*, and *Wolinella recta*. Periodontal microorganisms were isolated from periodontal lesions of dental patients. Bacteria were identified, purified and grown to late logarithmic phase in basal broth. Cells were then pelleted, lyophilized, and LPS extracted by the hot phenol-water method of Westphal and Jann (76). *Wolinella recta* LPS was prepared by the method of Darveau and Hancock (10). Protein content of the LPS preparations was assayed by the method of Lowry (35) using fraction V bovine albumin (Pentax) as protein standard.

Assay for PGE₂: Levels of PGE₂ in the culture supernatants were assayed by radioimmunoassay using absorption to dextran-coated charcoal. Anti-PGE₂ antibody and ³H-PGE₂ were purchased from Seragen and New England Nuclear, respectively. The anti-PGE₂ antibody demonstrates 100% cross-reactivity with PGE₁, however, other investigations in this laboratory have demonstrated that the E series prostaglandin released from human counterflow isolated monocytes consists of >97% PGE₂ when assayed by gas chromatography-mass spectrometry (manuscript submitted). The PGE₂ levels in culture supernatants were extrapolated from PGE₂ standard binding utilizing logit-log transformation.

Data Analysis: Data are represented in Tables 1 and 2 as ng/ml of PGE₂ in culture supernatants. Differences between treatment means were tested by the independent-samples paired t-test. Data presented are from 6 experiments except where indicated.

RESULTS

Salmonella, *B. intermedius*, and *B. gingivalis* LPS preparations were found to contain 5.1 %, 6.9%, 11.9% protein, respectively, as measured by Lowry assay. Protein was undetectable in *Wolinella* and *A. a.* LPS preparations.

Table 1 demonstrates the amounts of PGE₂ released during three consecutive 24 hour intervals from monocytes treated with *Salmonella*, *A. a.*, *B. intermedius* and *B. gingivalis* LPS preparations. *Salmonella* was the most potent LPS tested over all three time intervals resulting in approximately two-fold more PGE₂ release than *B. intermedius* and *A. actinomycetemcomitans* and seven-fold more than *B. gingivalis*. Lipopolysaccharides from *B. intermedius* and *A. a.* demonstrated similar abilities to stimulate PGE₂ release during the 0-24 hour interval, however, *A. a.* stimulated release was significantly greater than *B. intermedius* for the 24-48 and 48-72 hour intervals. *A. a.* and *B. intermedius* LPS stimulated PGE₂ release were approximately three to four times the release found with *B. gingivalis* which only differed significantly from controls during the initial 24 hour interval. Of interest was the finding that the differences found with *B. intermedius* and *gingivalis* were not significantly different. It can also be noticed that the amount of PGE₂ release decreased over later time intervals with all LPS preparations, so that by 72 hours *Salmonella* was the only LPS which resulted in significant stimulation of PGE₂ release over control medium.

Table 2 demonstrates the results from experiments which examined the relative potency of *Wolinella recta* LPS. *A. a.* and *Salmonella* were used as reference LPS preparations. The data indicate that *Wolinella* was the most

TABLE 1.

PGE₂ RELEASE (ng/ml)

| LPS (10ug/ml) | Time intervals (hours) | | |
|-----------------------|-----------------------------------|---------------------------------|--------------------------------|
| | 0 -24 | 24 - 48 | 48 - 72 |
| | Mean \pm SEM | Mean \pm SEM | Mean \pm SEM |
| Control | .30 \pm .16 | .14 \pm .05 | .07 \pm .02 |
| <i>Salmonella</i> | A5.64 \pm 1.17 ^{a,c,d} | A3.64 \pm 1.12 ^{a,d} | B .87 \pm .31 ^{a,d} |
| <i>A. a.</i> | A3.18 \pm .65 ^{b,c,d} | A1.51 \pm .38 ^{b,d} | .21 \pm .07 ^{b,c} |
| <i>B. intermedius</i> | A2.55 \pm .63 ^{a,b} | B .99 \pm .33 ^{a,b} | .19 \pm .07 ^b |
| <i>B. gingivalis</i> | A .78 \pm .20 ^{a,b} | .32 \pm .16 | .06 \pm .02 ^a |

A = p < .01 vs Control

a = p < .05 vs *A.a.*c = p < .05 vs *B. gingivalis*

B = p < .05 vs Control

b = p < .05 vs *Salmonella*d = p < .05 vs *B. intermedius*

Data are the means \pm SEM for 6 experiments except for *B. gingivalis* LPS which represents 5 experiments. Statistical test is paired students T-test.

TABLE 2.

PGE₂ RELEASE (ng/ml)

| LPS (10ug/ml) | Time intervals (hours) | | |
|-------------------|---------------------------------|----------------------------------|---------------------------------|
| | 0 - 24 | 24 - 48 | 48 - 72 |
| | Mean \pm SEM | Mean \pm SEM | Mean \pm SEM |
| Control | 3.06 \pm .93 | 2.10 \pm .71 | .24 \pm .04 |
| <i>Salmonella</i> | A7.01 \pm 1.68 ^a | B 5.18 \pm 1.56 ^{a,c} | 3.09 \pm 1.21 ^c |
| <i>A. a.</i> | B4.32 \pm 1.24 ^{b,c} | 2.76 \pm 1.01 ^{b,c} | B .88 \pm .27 ^c |
| <i>Wolinella</i> | B6.94 \pm 1.95 ^a | A 8.82 \pm 1.20 ^{a,b} | A7.30 \pm 1.17 ^{a,b} |

A = p < .01 vs Control

a = p < .05 vs *A.a.*

B = p < .05 vs Control

b = p < .05 vs *Salmonella*c = p < .05 vs *Wolinella recta*

Data are the means \pm SEM for 3 experiments. Statistical test is paired students t-test.

potent LPS tested. During the 0-24 hour interval *Wolinella* and *Salmonella* lipopolysaccharides had stimulated similar amounts of PGE₂ release, however, over the 24- 48 and 48-72 hour intervals the *Salmonella* response decreased while *Wolinella* increased to a point where the differences were statistically significant. It is important to note that the control release in this set of experiments was higher than that found in Table 1. However, the relative differences between the *Salmonella* and *A. a.* were consistent.

DISCUSSION

In the present study, we have compared the relative potencies of the lipopolysaccharides extracted from *B. gingivalis*, *B. intermedius*, *Wolinella recta*, and *A. actinomycetemcomitans* in the ability to stimulate the release of PGE₂ from human monocytes. *Salmonella* LPS was used as a reference, since its structure and potency have been extensively characterized (16). According to our results, the LPS from *Wolinella* was consistently more potent than that of the other periodontal organisms examined. Furthermore, the *Wolinella* LPS response was sustained over all three 24 hour intervals while a diminished response was observed with all other preparations over time. In contrast, the LPS prepared from *B. gingivalis*, *B. intermedius*, and *A. a.* were all shown to be less potent than either *Salmonella* or *Wolinella*. This finding is consistent with previous studies which demonstrated *Bacteroides* LPS to be weak relative to *Salmonella* in the stimulation of osteoclast-mediated bone resorption (24), the localized Schwartzman reaction (36,69), limulus lysate gelation (36), and pyrogenicity (41). In a study by Kiley and Holt (26), LPS from *A. a.* was also shown to be less potent than *Salmonella* when bone resorption activities were compared.

The differences observed between the LPS preparations, examined in this study, may result from structural and chemical differences between each preparation. According to several reports, *Bacteroides* possesses a unique LPS in that it lacks the acid labile keto-deoxyoctonic acid (KDO) and other heptose residues characteristic of enterobacterial LPS preparations (36,41,69). *A. a.*, on the other hand, has been reported to contain KDO residues, indicating that the relatively weak potency of this LPS preparation may be related to other factors (4). Accordingly, the fatty acid composition of *A. a.* has been shown to be substantially different from *Salmonella* LPS (4). The chemical composition of *Wolinella* LPS has not yet been characterized. Although structural differences may contribute to the relative potencies of LPS preparations, the complexity and heterogeneity of LPS preparations makes analyses of structure versus function highly speculative. Furthermore, the effect of the different extraction procedure used to prepare *Wolinella* LPS has not been established. However, a more detailed analysis of *Wolinella* LPS structure appears to be warranted in light of its high potency in stimulating PGE₂ release.

Protein contaminants associated with LPS preparations may participate in monocyte activation processes. Consistent with previous reports characterizing lipid A-associated protein in *E. coli* and *Salmonella* LPS preparations (40,67), our results demonstrate that *Bacteroides* and *Salmonella* LPS contain detectable levels of protein. Although the effect of lipid A-associated protein on monocyte secretory function remains to be established, protein contaminants are undoubtedly associated with LPS and may contribute to solubility and diffusion characteristics of LPS at disease sites. Furthermore, it is unlikely that LPS released from bacterial cell walls is free of protein contaminants from the cell wall. Additional work is required to assess these possibilities.

Although the 10 ug/ml dose of LPS was the only dose reported in the present study, dose response characteristics have been examined for each LPS preparation at 1 ng/ml, 100 ng/ml, and 10 ug/ml concentrations. All the LPS preparations demonstrated increased responses with increased doses of LPS with 10 ug/ml being the most potent. However, at the 1 ng/ml dose, only *Salmonella* and *Wolinella* LPS stimulated PGE₂ release to a greater extent than controls.

In this report, we have demonstrated that all LPS preparations tested stimulate the release of PGE₂ from human monocytes. That this process may occur in gingival connective tissue is strongly suggested by the ability of LPS to penetrate sulcular epithelium (59), the increased numbers of monocytes in periodontally diseased tissues (58), and the elevated levels of PGE₂ in periodontal tissues (18,34,38) and crevicular fluid (44) associated with diseased sites. The sustained potency of *Wolinella* LPS for up to 72 hours was a unique finding. Recent work in our laboratory has indicated that PGE₂ release from LPS stimulated monocytes may be potentiated by pretreatment with gamma interferon (42), a product of T lymphocytes. Furthermore, we have found that gamma interferon cotreatment with LPS results in sustained and potentiated PGE₂ release from monocytes treated with *Bacteroides* LPS (Chapter 3). Surprisingly, under identical conditions, *Salmonella* LPS was only slightly affected by gamma interferon. The apparent sustained potency of *Wolinella* LPS, in the absence of gamma interferon, demonstrates a potentially significant mechanism by which this LPS may be more pathogenic. Further studies are in progress to examine this possibility.

CHAPTER 2

IFN- γ potentiation of lipopolysaccharide-induced eicosanoid release from
human monocytes.

ABSTRACT

Interferon- γ (IFN- γ) can act to potentiate lipopolysaccharide (LPS)-stimulated processes in mononuclear phagocytes, including interleukin-1 release and tumoricidal activity. The present investigation examined the capacity of (IFN- γ) to modulate LPS-stimulated prostaglandin E₂ (PGE₂) and thromboxane B₂ (TxB₂) release from counterflow isolated human monocytes. The release of PGE₂ and TxB₂ was compared for cells incubated with IFN- γ prior to treatment with LPS and for cells treated simultaneously with IFN- γ and LPS. Treatment of cells with IFN- γ prior to stimulation with LPS (10 μ g/ml, *Salmonella typhimurium*) resulted in elevated prostaglandin E (by immunoassay) and [³H] PGE₂ release from monocytes when compared with cultures treated with LPS alone. In contrast, IFN- γ pretreatment did not potentiate labeled or immunoreactive TxB₂ release from LPS-treated monocytes. IFN- γ pretreatment without LPS stimulation did not result in elevated eicosanoid release over controls. In addition, continuous treatment of monocytes with both IFN- γ and LPS did not result in greater release PGE₂ and TxB₂ than the summed individual effects of IFN- γ and LPS. These results indicate that IFN- γ selectively potentiates LPS stimulated arachidonic acid conversion to PGE₂ and not TxB₂ in human monocytes. This effect was observed only for monocytes pretreated with IFN- γ prior to stimulation with LPS.

INTRODUCTION

Recent evidence has demonstrated the capacity of interferon- γ (IFN- γ) to potentiate bacterial lipopolysaccharide (LPS)-stimulated secretory responses in monocytes and macrophages, such as the secretion of interleukin-1(2). Although LPS is recognized as a potent stimulator of eicosanoid release from

monocytes and macrophages, little is known concerning the effect of IFN- γ on LPS-mediated eicosanoid release from monocytes. Previous investigations have generally characterized prostaglandin release by pretreating cells with IFN- γ prior to activating with a secondary stimulus (3,22). High doses of IFN- γ (1,000 U/ml) have been shown to increase prostaglandin E₂ (PGE₂) release from macrophages treated with phorbol myristate acetate (PMA) and A-23187 but not zymosan (3). In contrast, IFN- γ (1U/ml) was reported to be ineffective in promoting elevated release of PGE₂ following treatment of macrophages with PMA (22). From these results, IFN- γ demonstrates a variable capacity to potentiate prostaglandin release from stimulated macrophages depending on IFN- γ dose and the secondary stimulus. However, initial exposure to IFN- γ followed by a secondary stimulus may have little relevance to the process of macrophage activation in the pathogenesis of chronic inflammation. Instead, macrophages recruited to sites of LPS-elicited chronic inflammation are likely to be stimulated simultaneously with IFN- γ released locally from activated T lymphocytes. The present investigation, therefore, compared the effects of simultaneous and sequential treatment with IFN- γ on LPS-mediated PGE₂ release from monocytes.

Human monocytes stimulated with LPS release other cyclooxygenase-derived products of arachidonic acid, such as thromboxane B₂ (TxB₂) (56). Furthermore, release of TxB₂ is quantitatively greater than PGE from counterflow-separated monocytes during early phases of treatment with LPS (57). Because TxB₂ and PGE₂ are derived from a common substrate, arachidonic acid, and are released in elevated amounts from monocytes treated with LPS, it was of interest to examine IFN- γ effects on TxB₂ release in addition to PGE₂.

METHODS AND MATERIALS

Materials : [$^3\text{H}_8$] Arachidonic acid (sp. act., 83.8 Ci/mmol), [$^3\text{H}_8$] TxB_2 and [$^3\text{H}_7$] PGE_2 were obtained from New England Nuclear. PGE_2 2-*p*-toluidinylnaphthalene 6-sulfonate, penicillin, and streptomycin were obtained from Sigma Chemical Co. Anti- PGE_2 and anti- TxB_2 were obtained from Seragen and defined fetal calf serum was obtained from Hyclone Labs. Bacterial lipopolysaccharide (*Salmonella typhimurium*) was obtained from Difco Laboratories and recombinant human IFN- γ was obtained from Amgen Biochemicals.

Cell Isolation and Culture : Citrated fresh human blood, obtained by venipuncture, was separated by Ficoll-Hypaque density gradient centrifugation to yield a mononuclear cell fraction. This fraction was then subjected to counterflow centrifugation (53), during which platelets and lymphocytes were removed to yield a highly enriched population of monocytes, as previously described (56). Monocytes were then pelleted in Ca-Mg-free phosphate-buffered saline with 1% autologous plasma.

The cells were then resuspended in a known volume of MCDB 104 medium (37) containing 1% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, and monocyte recovery was determined by electronic counting (Coulter Electronics). The cell suspension was then diluted to achieve a concentration of 1.0×10^6 cells/ml, and 1-ml aliquots were placed in plastic (Falcon, 16-mm) culture dishes. All incubations were carried out at 37°C in the presence of 5% $\text{CO}_2/95\%$ air. After 2 h, medium was exchanged twice to remove nonadherent cells, and culture treatments were initiated. For experiments evaluating labeled metabolite release, labeled arachidonic acid was added to culture medium under conditions described in the Results section.

RIA for PGE₂ and TxB₂ : PGE and TxB₂ levels in culture supernatants were determined by immunoassay utilizing competitive adsorption to dextran-coated charcoal (according to Seragent technical bulletin). All determinations were carried out in duplicate and the average counts for each pair used to calculate PGE and TxB₂ levels. Regression coefficients for logit-log-transformed standard curves were generally greater than 0.990. Because the anti-PGE₂ antibody demonstrated 100% cross-reactivity with PGE₁ at 50% bound/total, immunoassay results were described in terms of ng/ml PGE released.

Labeled Arachidonic Acid Metabolite Extraction and Separation : Arachidonic acid metabolites were extracted by first adjusting the pH of culture supernatants to 3.5 with formic acid (70%) followed by 3 X 3-ml washes with ethyl acetate. The combined organic extracts were evaporated to dryness under vacuum and stored at -20° C under nitrogen. Prior to thin-layer chromatography, arachidonic acid (5 mg), PGE₂ (5 mg) and TxB₂ (5 mg) were added to each sample and the contents dried under a stream of nitrogen. Individual samples were dissolved in 10 ml of chloroform, vortexed, and spotted on silica gel G plates (precoated 200 mm, LHP-K plates, Whatman). This was followed by three additional washes with 5 ml of chloroform, spotting each wash immediately below the previous application. Plates were then developed in one dimension with chloroform/methanol/acetic acid/H₂O(90:8:1:0.8, vol/vol) (71). After drying, plates were sprayed with fluorescent indicator (1mM 2-*p*-toluidinylnaphthalene 6-sulfonate in 50 mM Tris, pH 7.4) and the lipid spots visualized with ultraviolet light. Arachidonic acid metabolites were identified by comigration with authentic standards (R_f values for arachidonic acid, 0.80 ± 0.02 ; TxB₂, 0.40 ± 0.01 ; and PGE₂, 0.512 ± 0.01 , n = 4), after which spots were scraped and counts determined.

Determination of Cell DNA Content : DNA content of adherent monocytes was determined after harvesting culture medium at 72 h. Cells were treated with 0.5 ml of 5 mM NaOH and sonicated briefly. After a 24-h period, 100 μ l was assayed for DNA content by the 4',6-diamidino-2-phenylindole fluorometric assay of Brunk *et al* (5).

RESULTS

Table 1 demonstrates the release of immunoreactive PGE from human monocytes treated simultaneously with LPS and IFN- γ . As shown, the release of PGE is maximally stimulated during the 24 to 48 h culture period followed by a modest decrease in the rate of release. IFN- γ had no significant effect on PGE release when administered alone or together with LPS. DNA levels recovered at 72 h from monocyte cultures were as follows: 7.41 ± 0.25 , 10.18 ± 0.15 , 9.53 ± 0.93 , and 8.35 ± 0.15 ng/culture well for control, IFN- γ -, LPS-, and LPS + IFN- γ -treated cultures, respectively.

TABLE 1. PGE RELEASE FROM MONOCYTES TREATED WITH IFN- γ AND LPS

| | PGE (ng/ml) | | | |
|-------------|-----------------|-----------------|------------------|---------------------|
| | Control | IFN- γ^b | LPS ^a | IFN- γ + LPS |
| 0-8 hours | 0.38 ± 0.15 | 0.63 ± 0.24 | 2.89 ± 0.16 | 3.04 ± 0.38 |
| 8-24 hours | 1.27 ± 0.40 | 1.19 ± 0.39 | 3.62 ± 0.72 | 2.70 ± 0.33 |
| 24-48 hours | 0.30 ± 0.30 | 0.84 ± 0.67 | 5.79 ± 1.95 | 5.80 ± 1.58 |
| 48-72 hours | 0.27 ± 0.09 | 0.74 ± 0.23 | 3.94 ± 1.10 | 4.86 ± 0.75 |

Data represent the mean \pm standard error for 6 determinations

Monocytes were inoculated at 10^6 cells/ml in 16mm wells and treated with indicated media. Media were exchanged at 8, 24, and 48 hrs and replaced with identical fresh media.

^a 10 μ g/ml LPS (*Salmonella typhimurium*)

^b 10 U/ml IFN- γ .

Table 2 demonstrates the release of immunoreactive PGE from monocytes cultured for 48 h with or without IFN- γ followed by treatment with control medium or LPS (10 μ g/ml) for an additional 24-h period. Control cultures demonstrated peak PGE release during the 8 to 24 h time interval with diminished release thereafter. Treatment of monocytes at 48 h with LPS produced a twofold stimulation of PGE release over controls. IFN- γ (10 U/ml) alone produced no significant effect on PGE release when compared with control cultures. IFN- γ was not included in culture medium from 48 to 72 in these experiments. Finally, treatment of monocytes with LPS after 48 h of exposure to IFN- γ treatedmodul in a 10-fold stimulation of PGE release over IFN- γ treated cultures and a sevenfold stimulation over LPS-treated cultures. DNA levels recovered at 72 h from monocyte cultures were as follows: 4.45 ± 0.18 , 6.93 ± 0.27 , 5.71 ± 0.21 , and 7.84 ± 0.48 ng/culture well for control, IFN- γ , LPS- and IFN- γ + LPS-treated cultures, respectively.

TABLE 2. PGE RELEASE FROM MONOCYTES TREATED WITH IFN- γ FOLLOWED BY LPS
PGE (ng/ml)

| Time Interval | Control | IFN- γ (10 U/ml) | | |
|---------------------|-----------------|-------------------------|-----------------|---------------------|
| 0-8 hr. | 0.55 ± 0.17 | 0.75 ± 0.36 | | |
| 8-24 hr. | 2.01 ± 0.12 | 1.67 ± 0.13 | | |
| 24-48 hr. | 0.86 ± 0.13 | 0.84 ± 0.35 | | |
| Treatment at 48 hr. | Control | LPS (10 μ g/ml) | IFN- γ | LPS (10 μ g/ml) |
| 48-72 hr. | 0.29 ± 0.02 | 0.70 ± 0.18 | 0.56 ± 0.23 | 5.15 ± 1.04 |

Data represent the mean \pm standard error for 4 experiments

Monocytes plated as in Table 1. Where indicated, cells were treated with IFN- γ from 0-48 hrs. LPS treatment occurred during the 48-72 hr interval. Medium was exchanged at the end of each interval.

Table 3 demonstrates the release of labeled PGE₂ and TxB₂ from monocytes labeled with [³H]-arachidonic acid. Labeling with [³H]-arachidonic acid occurred while cells were adhering to culture dishes (2 h), after which unincorporated label was removed through rinsing and cells treated with the indicated stimulants. IFN- γ alone (10 U/ml) did not stimulate the release of labeled PGE₂ compared with controls and only slightly elevated labeled TxB₂ release. Continuous treatment of monocytes with LPS resulted in fourfold stimulation of labeled PGE₂ release which diminished to approximately twofold by 72 h. Threefold stimulation of labeled TxB₂ release was observed during the 0 to 8 h interval and no stimulation over controls thereafter. Continuous treatment with LPS and IFN- γ did not elevate PGE₂ release over LPS alone and only slightly elevated TxB₂ release. However, IFN- γ pretreatment followed by LPS resulted in twofold stimulation of PGE₂ release over cells not pretreated with IFN- γ and sixfold stimulation over cells treated with IFN- γ alone. TxB₂ release was elevated approximately threefold in cultures treated with LPS and was not affected by IFN- γ pretreatment.

The time course of PGE₂ and TxB₂ release during the 48 to 72 h culture period are demonstrated in Fig. 1. For labeled eicosanoid release, monocytes were labeled with [³H]arachidonic acid during the first 24 h of culture. Cells were then rinsed with unlabeled fresh medium and treated with either control medium or medium containing 10 U/ml IFN- γ . At 48 h, monocyte cultures were treated as shown and medium exchanged at 50, 56, and 64 h and harvested at 72 h. Therefore, each histogram bar represents metabolite release only during the indicated interval. Cultures treated with IFN- γ followed by LPS demonstrated potentiated PGE and PGE₂ release when compared with cultures treated only with LPS. IFN- γ potentiation of prostaglandin release was maximal during the 64 to 72 h interval. IFN- γ alone, did not elevate PGE

Table 3. EFFECT OF IFN- γ TREATMENT ON PGE₂ AND TxB₂ RELEASE FROM LPS-TREATED MONOCYTES

| <i>Labelled eicosanoid release (cpm / sample)</i> | | | | |
|--|----------------------|-------------------|--------------------|--------------------|
| | <i>Time Interval</i> | | | |
| | <i>0-8 hours</i> | <i>8-24 hours</i> | <i>24-48 hours</i> | <i>48-72 hours</i> |
| PGE₂ | | | | |
| Control | 220 \pm 42 | 239 \pm 4 | 102 \pm 19 | 25 \pm 9 |
| Control (0-48 hr.) + LPS (48-72) | | | | 77 \pm 47 |
| IFN- γ | 206 \pm 22 | 234 \pm 20 | 119 \pm 25 | 27 \pm 15 |
| IFN- γ (0-48 hr.) + LPS (48-72) | | | | 151 \pm 40 |
| LPS (0-72 hr.) | 807 \pm 46 | 318 \pm 10 | 215 \pm 7 | 41 \pm 33 |
| LPS + IFN- γ (0-72 hr.) | 862 \pm 86 | 410 \pm 10 | 288 \pm 59 | 44 \pm 31 |
| TxB₂ | | | | |
| Control | 684 \pm 99 | 614 \pm 73 | 157 \pm 25 | 56 \pm 19 |
| Control (0-48 hr.) + LPS (48-72) | | | | 140 \pm 77 |
| IFN- γ | 763 \pm 73 | 748 \pm 96 | 188 \pm 46 | 46 \pm 40 |
| IFN- γ (0-48 hr.) + LPS (48-72) | | | | 149 \pm 53 |
| LPS (0-72 hr.) | 2043 \pm 241 | 611 \pm 19 | 145 \pm 9 | 42 \pm 41 |
| LPS + IFN- γ (0-72 hr.) | 2343 \pm 27 | 721 \pm 4 | 261 \pm 41 | 70 \pm 28 |

Data represent the mean + standard error for 4 determinations

Cells from two donors were labeled with 0.1 μ Ci/ml [³H]arachidonic acid during cell adherence to culture dishes (2 h) after which the cells were washed twice with unlabeled medium. Treatment of cultures with the indicated agents was initiated at this time (indicated as time 0) and medium was exchanged at 8, 24, and 48 hrs. Each determination represents labeled eicosanoid extracted from replicate pooled culture supernatants.

release over untreated controls. IFN- γ pretreatment also did not markedly affect LPS stimulation of labeled and immunoreactive TxB₂ release. In addition, LPS-stimulated TxB₂ release diminished almost to control levels during the 64 to 72 h interval, whereas, PGE₂ release remained at an elevated level with IFN- γ treatment. DNA recovered at 72 h from monocyte cultures was as follows: 4.99 ± 0.41 , 5.53 ± 0.56 , 6.34 ± 0.77 , and 6.60 ± 0.92 ng/culture well for control, IFN- γ -, LPS-, and IFN- γ + LPS-treated cultures, respectively.

Figure 2 demonstrates the effect of varying concentrations of IFN- γ on [³H]PGE₂ and immunoreactive PGE release from LPS- (10 mg/ml) treated monocytes. Labeled PGE₂ release was elevated over controls by approximately 10-fold with 1,000 U/ml IFN- γ , whereas, immunoreactive PGE release was elevated by approximately 50-fold. IFN- γ at concentrations of 1 U/ml or less demonstrated no potentiation of LPS-mediated prostaglandin release. The minimum dose of IFN- γ that elevated PGE₂ release was 10 U/ml. Relative to [³H]PGE₂, release of [³H]TxB₂ was only moderately stimulated with higher doses of IFN- γ (data not shown in Fig. 2). Labeled TxB₂ release was 807 ± 69 , 664 ± 15 , and 291 ± 38 cpm/2ml for 1,000, 100, and 10U/ml IFN- γ , respectively. IFN- γ doses of 1 U/ml or less did not promote elevated [³H]TxB₂ release over cultures treated with LPS alone (137 ± 24 cpm/2ml). As with PGE₂ release, 10 U/ml IFN- γ was the minimum dose that elevated TxB₂ release. DNA levels recovered from control cultures were 10.83 ± 0.74 ng/culture well whereas cultures pretreated with the lowest to highest levels of IFN- γ were as follows: 14.29 ± 0.86 , 10.72 ± 1.32 , 10.63 ± 1.41 , 5.22 ± 1.43 , 7.89 ± 2.72 , respectively.

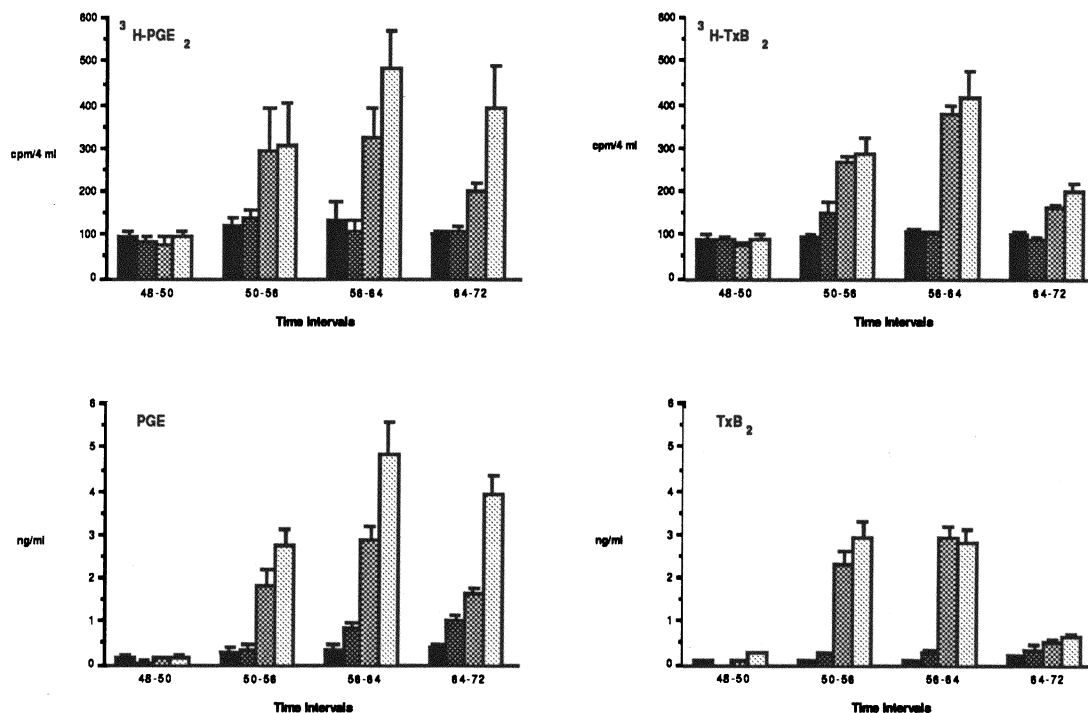


Figure. 1 Time course of PGE₂ and TxB₂ release from LPS-treated monocytes. For [³H] eicosanoid release, monocytes were labeled from 0-24 hours with 0.1μCi/ml [³H]arachidonic acid. Cells then received the following treatments: (i) Control medium (24-72 h) ■, (ii) 10 U/ml IFN-γ (24-48 h) followed by control medium (48-72 h) ■, (iii) control medium (24-48 h) followed by 10 mg/ml LPS (48-72 h) ▨, (iv) 10 U/ml IFN-γ (24-48 h) followed by 10 mg/ml LPS (48-72 h) ▩. Medium was exchanged at 48, 50, 56, 64, and 72 h. Histogram bars represent metabolite release during the indicated intervals. Results are expressed for eight trials from three experiments. PGE₂ and PGE release from IFN-γ + LPS-treated cultures were significantly elevated over LPS-treated cultures (by t-statistic for two means: a, $p < 0.025$; b, $p < 0.05$).

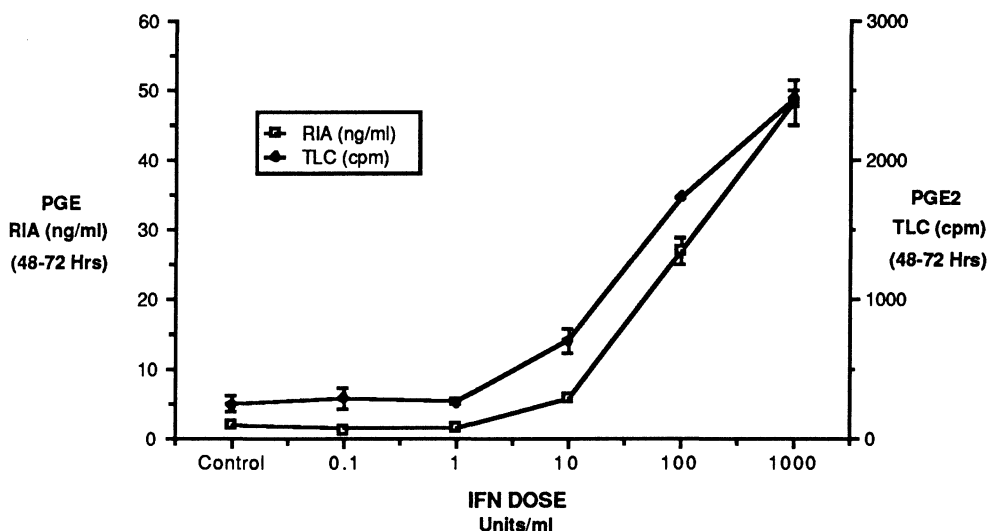


Figure 2. Effect of increasing levels of IFN- γ on LPS-mediated release of PGE₂. Monocytes were labeled from 0-24 h with 0.1 μ Ci/ml [³H]arachidonic acid followed by rinsing to remove unincorporated label. Cells were then treated with IFN- γ at the indicated levels from 24-48 hrs followed by replacement with control medium or medium containing LPS (10 μ g/ml). Metabolite release was measured only for the 48 -72 h media samples. [³H] PGE₂ and PGE release are shown as the mean \pm standard error for two trials. Treatment of monocytes with IFN- γ alone did not significantly elevate PGE₂ or PGE release over control cultures during the 48-72 h interval.

DISCUSSION

This investigation demonstrates the importance of simultaneous versus sequential treatment with IFN- γ and LPS in promoting PGE₂ release from human monocytes. Monocytes treated continuously with IFN- γ and LPS released only slightly greater levels of labeled PGE₂ (Table 3) than monocytes treated with LPS alone. Release of immunoreactive PGE (Table 1) under identical treatment conditions was not affected in a consistent manner. Because IFN- γ alone slightly elevated PGE₂ release from human monocytes, these results could reflect an additive effect of the two agents. However, preexposure to IFN- γ for 24-48 h followed by LPS treatment resulted in a pronounced increase in PGE₂ release, whereas, IFN- γ alone had no effect

during the same time period. These results cannot be reconciled on the basis of a simple additive effect of the two agents and indicate that IFN- γ acts to modify monocyte secretory responses through different mechanisms than LPS. Although emerging evidence now suggests that IFN- γ modulates protein kinase C activity (21), there is little evidence reported concerning the mechanisms by which LPS and IFN- γ mediate alterations in monocyte secretory function.

As noted with PGE₂ release, [³H]TxB₂ release with continuous IFN- γ and LPS treatment was slightly elevated over LPS alone. In contrast to PGE₂, labeled and immunoreactive TxB₂ release were not potentiated with IFN- γ pretreatment. This finding indicates that 10 U/ml IFN- γ can selectively stimulate the conversion of arachidonic acid to PGE₂ following treatment with LPS. However, higher doses of IFN- γ were shown to promote LPS-mediated TxB₂ release but not to the same extent as labeled PGE₂ release. Our results are in contrast to a recent report demonstrating unchanged or slightly elevated thromboxane release and reduced PGE₂ release from *Listeria monocytogenes*-elicited macrophages compared with resident macrophages (70). Sustained TxB₂ synthesis from *Listeria*-elicited macrophages was proposed to result from IFN- γ interaction with elicited cells. Our results clearly indicate that IFN- γ selectively potentiates PGE₂ release over TxB₂ release from LPS-treated human monocytes.

After 72 h in culture, DNA levels recovered from adherent monocytes were found to vary, but not in a consistent pattern following specific treatments. Although all experiments were carefully controlled to assure inoculation of 1×10^6 cells/culture well, cell retention over 72 h was variable from donor to donor. Some variable cell adherence has been noted over a much larger group of experiments conducted in this laboratory. In most

cases, release of eicosanoids from monocyte cultures did not correlate with DNA levels recovered from cultures. Most striking was the increased cell adherence with lower doses of IFN- γ whereas higher doses of IFN- γ reduced cell adherence after LPS stimulation (Fig. 2). In contrast, PGE₂ release was markedly elevated only for cultures treated with higher doses of IFN- γ . This observation suggests that elevated eicosanoid release is regulated primarily by adherent monocytes and may reflect differential effects of IFN- γ on specific populations of monocytes.

The potentiation of PGE₂ release demonstrated by IFN- γ in this investigation was consistent with previously reported effects of IFN- γ on monocyte/macrophage function. IFN- γ has been shown to prime macrophages for LPS-mediated tumor cell killing (47), and IFN- γ can restore time-dependent reduction of interleukin-1 secretion from LPS-treated monocytes (2). In addition, the lack of a significant stimulation of PGE₂ release by monocytes treated with IFN- γ alone was also consistent with previous reports demonstrating that macrophage tumor cell killing and interleukin-1 release were not elevated by treatment with IFN- γ alone. A dose of 10 U/ml IFN- γ was utilized in most experiments since this dose has been shown to promote maximal tumor cell killing with mouse macrophages (47) and to exceed by one order of magnitude the dose necessary for maximal stimulation of arachidonic acid release from mouse macrophages treated with PMA (22). In the present investigation, IFN- γ levels greater than 10 U/ml demonstrated increased potential to promote LPS-mediated PGE₂ release. IFN- γ potentiation of LPS-mediated PGE₂ release was most prominent with sequential treatment conditions, suggesting that simultaneous exposure to IFN- γ and LPS in sites of chronic inflammation may not lead to significantly greater eicosanoid release than with LPS alone. It should be emphasized that

these results apply to *Salmonella typhimurium* LPS and may not reflect effects by other LPS preparations. Experiments in our laboratory are directed at further examining PGE₂ release from monocytes simultaneously treated with IFN- γ and other LPS preparations.

CHAPTER 3

Gamma interferon modulation of prostaglandin E₂ release from monocytes stimulated with lipopolysaccharides from *Bacteroides gingivalis*, *Bacteroides intermedius* and *Salmonella typhimurium*.

ABSTRACT

Lipopolysaccharides (LPS) from *Bacteroides* species have been shown to be relatively weak in comparison to that of enterobacteria such as *Salmonella*, when examined by pyrogenicity, bone resorption, and the localized Schwartzman reaction. This is contrary to the proposed pathogenic role of *Bacteroides* species in the etiology of diseases such as adult periodontitis. In this investigation, the relative potency of LPS from *Bacteroides gingivalis* and *intermedius* were compared to that of *Salmonella*, with respect to the stimulated release of PGE₂ from human monocytes. Furthermore, the effect of recombinant gamma interferon was examined for possible potentiation of the LPS response. Monocytes, separated by counterflow centrifugation, were exposed to LPS (1 ng/ml, 100 ng/ml and 10 ug/ml) alone or in combination with gamma interferon (10 Units/ml). PGE₂ levels in culture medium were determined by radioimmunoassay for medium samples exposed to monocytes over the following time intervals: 0-8, 8-24, 24-48, and 48-72 hours.

Although IFN- γ did not stimulate PGE₂ release alone, the amount of PGE₂ released from monocytes stimulated with *Bacteroides* species LPS was significantly increased when monocytes were cultured concomitantly with IFN- γ . PGE₂ release elicited by *Salmonella* LPS was not significantly affected by the addition of interferon. Differences were observed in the relative potency of the three LPS preparations tested; *Salmonella* LPS stimulation resulted in approximately 2-3 times the PGE₂ release as *Bacteroides* species LPS. This difference was minimized, however, with interferon cotreatment. It is concluded that gamma interferon can increase the release of PGE₂ from *Bacteroides* LPS-stimulated monocytes and that this effect can partially overcome the observed weakness of these LPS preparations.

INTRODUCTION

Numerous gram-negative bacteria, particularly the enterobacteria, possess lipopolysaccharides which are similar in general structure and endotoxic activity (52). However, LPS extracted from some of the *Bacteroidaceae*, particularly *Bacteroides intermedius* and *gingivalis*, lack particular chemical constituents commonly found in enterobacterial LPS (36,41,69). Furthermore, in tests of pyrogenicity (41), the localized Schwartzman reaction (36,69), limulus lysate gelation, chick embryo lethality (36), and bone resorption (24), LPS from *Bacteroides* species have been shown to be relatively weak in comparison to a standard enterobacterial LPS, such as *Salmonella typhimurium*. The apparent weakness of *Bacteroides* LPS does not support a significant role of LPS from these species in periodontal pathogenesis, yet these organisms are closely associated with progressive attachment loss in humans. Furthermore, enterobacterial lipopolysaccharides have been shown to stimulate the release of numerous biologically active substances from monocytes, particularly prostanoids and cytokines (2,17,55); however, investigations into *Bacteroides* LPS stimulation of prostanoid release have been lacking. Because prostaglandin E₂ (PGE₂) is a prominent secretory product of monocytes (55) which can mediate inflammatory and immune responses as well as bone resorption (17,65), one of the aims of this investigation was to compare and partially characterize the capacity of *B. intermedius* and *B. gingivalis* LPS preparations to stimulate PGE₂ release, relative to that of *Salmonella*.

Gamma interferon (IFN- γ) has been shown to produce a number of direct effects on monocytes, including increased expression of Fc receptors (20,49) and Ia antigen (27). Furthermore, interferon has the ability to modulate both tumoricidal activity (47) and interleukin 1 release (2) when

monocytes are treated with LPS. Although monocytes do not release IFN- γ , LPS-treated monocytes have been shown to elevate IFN- γ release from T lymphocytes (30). Therefore, LPS-mediated responses in monocytes are subject to modification by IFN- γ when stimulated in the presence of T lymphocytes. Such conditions are likely to exist with established lesions of chronic adult periodontitis. Although we have recently demonstrated that IFN- γ pretreatment greatly enhanced PGE₂ release from monocytes secondarily treated with *Salmonella* LPS (42), no data exists concerning IFN- γ potentiation of LPS stimulated PGE₂ release from monocytes simultaneously exposed to IFN- γ and LPS. Furthermore, examination of IFN- γ effects upon *Bacteroides* LPS stimulation have not been reported. Therefore, a second aim of this investigation was to characterize and compare the effects of IFN- γ on the release of PGE₂ from monocytes stimulated with LPS extracted from *Bacteroides intermedius* and *gingivalis*, in comparison to *Salmonella typhimurium*.

METHODS AND MATERIALS

Cell Separation : Monocytes were separated from whole venous blood of random human donors by ficoll-Hypaque sedimentation (400 x g x 35 min.) at 10°C. The mononuclear leukocyte rich fractions were collected, diluted, and washed two times in Ca⁺⁺ and Mg⁺⁺ free, phosphate buffered saline (PBS) (125 x g x 15 min.) at 10°C. The cells were then counted electronically (Coulter Electronics) and monocyte enrichment achieved by counterflow centrifugation (Beckman Instruments). Autologous plasma (1%) was then added and monocytes sedimented (125 x g x 15 min.) and resuspended in MCDB 104 medium (37) supplemented with 1% defined heat inactivated fetal calf serum (Hyclone), 100 U/ml penicillin and 100 mcg/ml streptomycin. Cells were counted and plated in plastic (Falcon Plastics) 16mm wells (10⁶ cells

per well). At 2 hours the cells were washed two times in medium to remove any non-adherent cells. Cultures were incubated at 37°C in an H₂O saturated 5% CO₂ atmosphere. Cell viability was routinely confirmed by trypan blue exclusion.

LPS and IFN-γ Preparations : LPS was obtained from the following bacterial strains: *Salmonella typhimurium* (Difco Laboratories), *Bacteroides gingivalis* and *Bacteroides intermedius* (kindly provided by Dr. Stan Holt, University of Texas Health Center at San Antonio). All LPS were prepared by the hot phenol-water method of Westphal and Jann (76). Recombinant IFN-γ was purchased from AMGEN Biochemicals with a specific activity of 1.6×10^7 Units/mg protein calibrated against NIH IFN-γ standard GG-23-901-530.

LPS Analysis : Protein content of the LPS preparations was assayed by the method of Lowry (35). Albumin (Fraction V, Pentax) was used as a standard. LPS preparations were subjected to SDS-PAGE in order to assess relative molecular weight of LPS associated proteins.

Experimental Conditions : Treatments consisted of the following: LPS (in concentrations of 1 ng/ml, 100 ng/ml, and 10 ug/ml), with or without IFN-γ (10 Units/ml), IFN-γ alone (10 Units/ml), and control medium. Media were exchanged at 8, 24, 48, and 72 hours and the supernatants assayed for PGE₂ content. Adherent cells retained in culture wells after the 72 hour media change were assayed for DNA content by the method of Brunk et al. (5). No significant differences were observed between the various treatments with respect to DNA levels.

Assay for PGE₂ : Levels of PGE₂ in the culture supernatants were assayed by radioimmunoassay using absorption to dextran-coated charcoal. Anti-PGE₂ antibody and ³H-PGE₂ were purchased from Seragen and New England Nuclear, respectively. The anti-PGE₂ antibody demonstrates 100%

cross-reactivity with PGE₁, however, other investigations in this laboratory have demonstrated that the E series prostaglandin released from human counterflow isolated monocytes consists of >97% PGE₂ when assayed by gas chromatography-mass spectrometry (manuscript submitted). The PGE₂ levels in culture supernatants were extrapolated from PGE₂ standard binding utilizing logit-log transformation.

Data Analysis : Differences between treatment means were tested by the independent-samples paired t-test. Data presented are from 3 experiments except where noted.

RESULTS

LPS analysis : *Salmonella*, *B. intermedius*, and *B. gingivalis* LPS preparations were found to contain 5.1 %, 6.9%, and 11.9% protein, respectively, as assessed by Lowry assay. LPS associated proteins were assessed by SDS-PAGE. Most contaminating proteins were of low relative molecular weight (< 20,000), particularly with the *B. intermedius* LPS. *Salmonella* LPS, purchased from Difco, contained a variety of protein fragments of relative molecular weights ranging from 60,000 to < 14,000.

Effect of IFN- γ on LPS stimulation of PGE₂ release : The addition of IFN- γ to LPS from either *Bacteroides* species resulted in greater release of PGE₂ than LPS alone, as shown in Figures 1 (*B. intermedius*) and 2 (*B. gingivalis*). Interferon treatment alone did not significantly stimulate PGE₂ release over controls, indicating that enhancement of PGE₂ release with interferon and LPS is not due to an additive effect of the two agents. Interferon potentiation was evident at all time intervals, except the 8-24 hour interval for *B. gingivalis*. During the 0-8 hour interval, the addition of 10 Units/ml of recombinant interferon to LPS (100 ng/ml and 10 ug/ml) from

B. gingivalis and *intermedius* resulted in significantly increased ($p < .05$) release of PGE_2 when compared to LPS alone. During the 8-24 hour interval, PGE_2 release stimulated by *B. intermedius* (100 ng/ml and 10 ug/ml doses) was significantly enhanced ($p < .01$ and $p < .05$, respectively) by $\text{IFN-}\gamma$. The greatest potentiation of PGE_2 release by interferon was exhibited with *Bacteroides* LPS during the 24-48 hour interval. All doses of *B. intermedius* and the 100 ng/ml and 10 ug/ml doses of *B. gingivalis* LPS resulted in significantly increased ($p < .01$) release of PGE_2 (compared to LPS alone) when interferon was added. The effect of interferon was still evident at the 48-72 hour interval although only at the 100 ng/ml and 10 ug/ml doses ($p < .05$) for both LPS preparations. It must be noted, however, that the amount of PGE_2 released from the 100 ng/ml dose of *B. gingivalis* LPS with interferon was not significantly different from that of interferon alone.

In contrast to *Bacteroides* LPS preparations, the addition of $\text{IFN-}\gamma$ had no significant effect on *Salmonella* LPS stimulated PGE_2 release (Figure 3), although the mean levels of PGE_2 released during the 48-72 hour interval appeared greater with interferon.

The modest potentiation of *Salmonella* LPS stimulation was examined further by evaluating the effect of increasing doses of interferon on LPS (10 ug/ml) stimulated PGE_2 release. Figure 4 demonstrates that higher doses of $\text{IFN-}\gamma$ tended to increase *Salmonella* LPS stimulation of PGE_2 release. Although maximal PGE_2 release was observed with 100 U/ml interferon, this

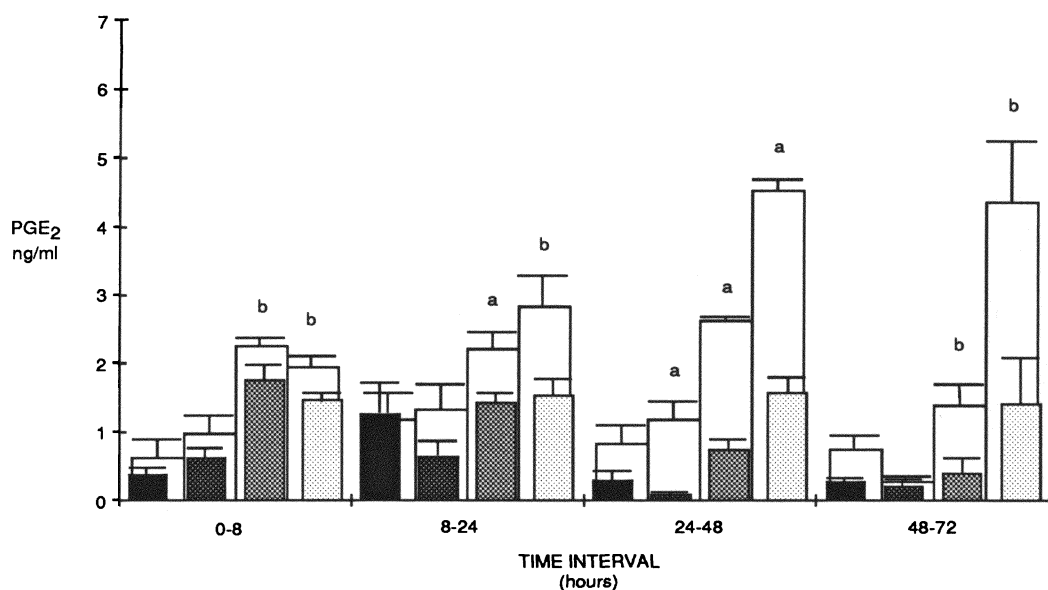


Figure 1. *Bacteroides intermedius* LPS

Legend for Figures 1-3

Fig. 1 (*Bacteroides intermedius*), Fig. 2 (*Bacteroides gingivalis*), and Fig. 3 (*Salmonella typhimurium*) illustrate amounts of PGE₂ (ng/ml) released, Mean \pm S.E.M. from 1×10^6 cells/ml exposed to the following doses of the respective LPS preparations over the indicated time intervals: 1 ng/ml , 100 ng/ml , 10 ug/ml , and control . The unfilled column superimposed behind each dose column represents data obtained from the respective dose of LPS with the addition of IFN- γ (10 Units/ml). a= p<.01 b= p<.05

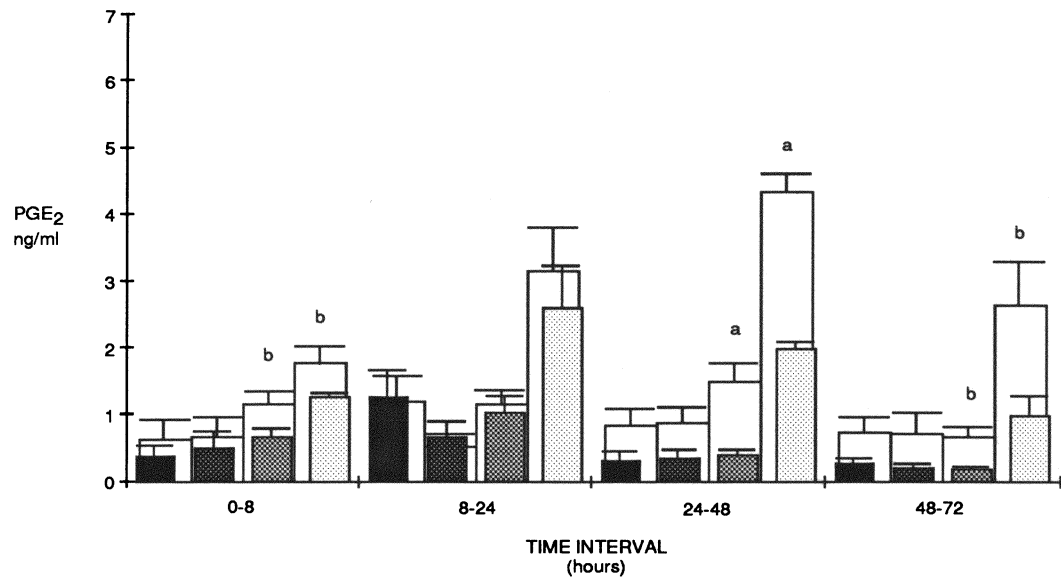


Figure 2. *Bacteroides gingivalis* LPS

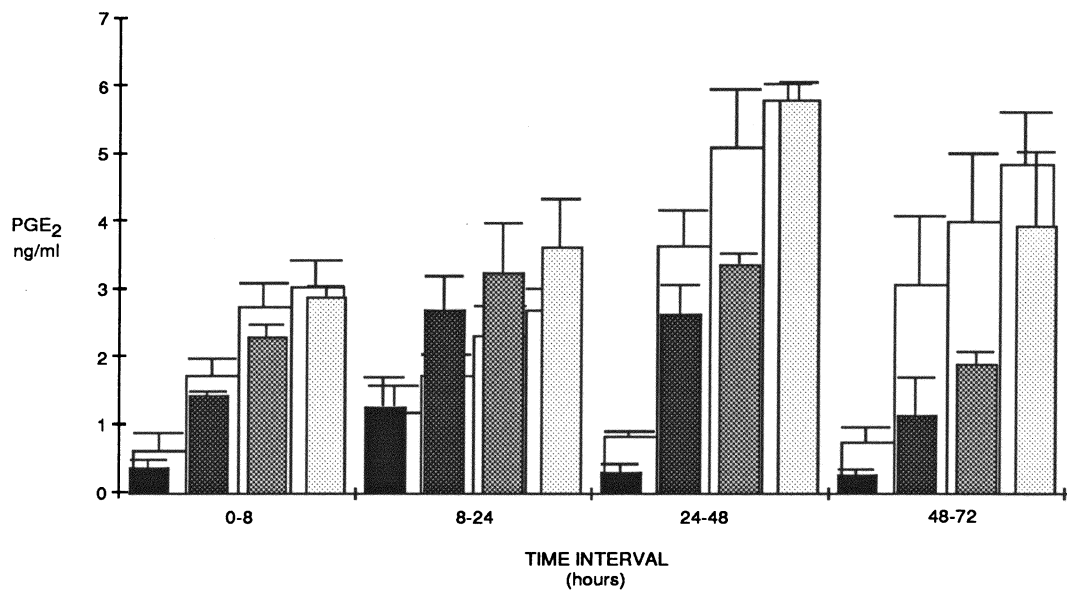


Figure 3. *Salmonella typhimurium* LPS

was associated with relatively high variability. In contrast, *B. gingivalis* LPS with 100 U/ml of interferon resulted in approximately 4-fold greater PGE₂ release compared to LPS alone. *Salmonella* LPS with 100 U/ml of interferon resulted in less than 2-fold increased PGE₂ release. It can also be noted that interferon alone had minimal effect on PGE₂ release.

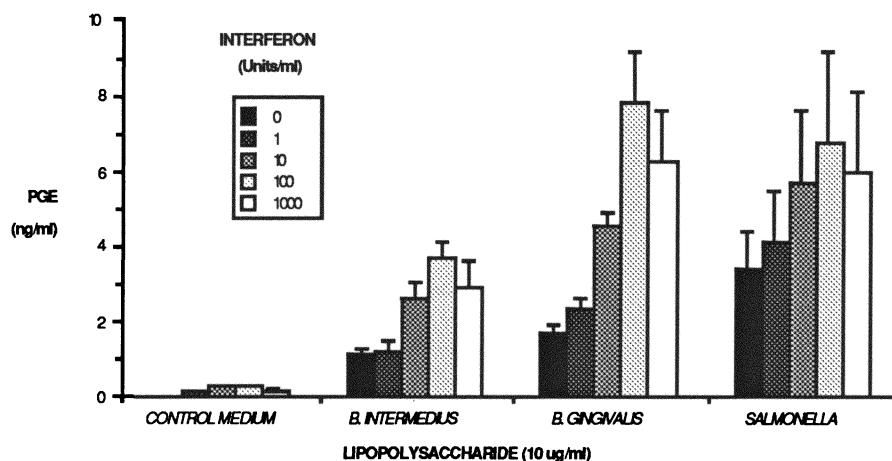


Figure 4. IFN- γ Dose response

Cells were treated with control medium, or LPS (10 ug/ml) from the three bacteria, and the indicated concentrations of IFN- γ at time 0. Identical media was exchanged at 24 hrs and experiment terminated at 48 hrs. Data depicts the mean \pm SEM of PGE₂ (ng/ml) released during the 24-48 hr time interval, data are from two experiments.

Capacity of LPS preparations to stimulate PGE₂ release : Salmonella (Figure 3) was the most potent LPS in stimulating PGE₂ release. At all doses and time intervals, treatment with *Salmonella* LPS resulted in greater PGE₂ release than either *Bacteroides* LPS preparation. In contrast, a 1 ng/ml dose of LPS from each *Bacteroides* species did not significantly stimulate PGE₂ release over controls. Stimulation with increasing doses of LPS from *Bacteroides* species resulted in dose responses which were reduced relative to *Salmonella* LPS.

When comparing lipopolysaccharides extracted from both *Bacteroides* species, similar stimulation of PGE₂ release was observed. However, 10 ug/ml was the only dose of *B. gingivalis* LPS which consistently resulted in PGE₂ release greater than controls. Also, the maximal or greatest effect of *B. gingivalis* and *intermedius* LPS occurred during the 8-24 hour interval and then gradually decreased, as opposed to that of *Salmonella* which attained maximal stimulation during the 24-48 hour interval before declining.

DISCUSSION

Unique to the experimental design of this study was the use of interferon and LPS concomitantly. This was thought to evaluate the effects of these agents as they would likely interact *in vivo* during inflammatory and/or immune responses. Previous reports have examined sequential activation of monocytes to a tumoricidal level (47) by first pretreating with interferon followed by administration of LPS. The same experimental design has been applied to the examination of interferon effects on various monocyte functions and have included different secondary stimuli, such as calcium ionophore, zymosan, immune complexes, phorbol diesters, and LPS (2,3,22). Although sequential activation of monocytes is relevant to the study of cell differentiation, it is not likely to accurately simulate microenvironmental conditions at sites of chronic inflammation, where cells are simultaneously exposed to many stimuli present in the extracellular environment. These differences were explored in a previous study which demonstrated that IFN- γ can potentiate PGE₂ release from monocytes with sequential treatment conditions but not with simultaneous treatment, using *Salmonella* LPS(42).

The experiments described herein have demonstrated two unique aspects of IFN- γ and LPS effects upon human monocyte release of prostaglandin E₂. First was the observed potentiation of PGE₂ release by simultaneous treatment with IFN- γ and LPS. That this effect was primarily associated with the LPS extracted from the *Bacteroides* species and not *Salmonella* was the second important observation. Furthermore, this study confirmed previous reports demonstrating the relative weak potency of *Bacteroides* LPS. It has been hypothesized that the absence of keto-deoxyoctonic acid and heptose in *Bacteroides* LPS may result in a structure with weak endotoxic potential (36). However, the relationship between the structure of *Bacteroides* species LPS and its biological activity has not been fully elucidated at this time.

Interferon potentiated the effects of *Salmonella* LPS to a minimal extent. This finding is in agreement with our previous study which showed minimal to no enhanced release of both radioimmunoassayable and ³H-labelled PGE₂ from monocytes treated simultaneously with LPS and 10 Units of IFN- γ (42). However, in the present study increased doses of IFN- γ were tested and shown to have greater enhancement of PGE₂ release.

When maximal doses of LPS were compared, interferon potentiation of *Bacteroides* LPS resulted in levels of PGE₂ release which were comparable to *Salmonella* LPS. This observation demonstrates a potentially significant mechanism for increasing the release of PGE₂ from cells exposed to a relatively weak LPS in combination with products of activated lymphocytes. The selective potentiation of *Bacteroides* LPS over *Salmonella* may reflect inherent structural differences between the preparations or a maximal stimulatory capacity of monocytes when exposed to interferon, regardless of the LPS preparation. Another possibility is that protein contaminants

associated with LPS preparations may participate in monocyte activation processes. Consistent with previous reports characterizing lipid A-associated protein in *E. coli* and *Salmonella* LPS preparations (40,67), our results demonstrate that *Bacteroides* LPS contains detectable levels of low molecular weight proteins ($< 20,000$) as assessed by SDS-PAGE. Lipid A-associated proteins have been observed to stimulate lymphocytes and macrophages isolated from LPS-hyporesponsive murine strain C3H/HeJ. Removal of these low molecular weight proteins from LPS preparations has been shown to render LPS preparations inactive in stimulation of cellular responses from C3H/HeJ cells. However, it has generally been observed that phenol-extracted LPS is free of these contaminating proteins while butanol-extracted LPS preparations are not. More importantly, the effect of these contaminating proteins on LPS stimulation of normal LPS-responsive murine cells has been shown to be relatively minor when comparing stimulation by butanol versus phenol-extracted LPS preparations(40,74). Although the effect of lipid A-associated protein on human monocyte secretory function remains to be established, protein contaminants are undoubtedly associated with LPS and may contribute to solubility and diffusion characteristics at disease sites. Additional work is required to assess these possibilities.

In the present study, interferon did not increase the sensitivity of monocytes to LPS since interferon effects were greater with high doses of LPS but little to no potentiation was observed with low doses of LPS. This finding is in conflict with several reports demonstrating an increased sensitivity of mononuclear phagocytes to a variety of stimuli following IFN- γ treatment (22,47). However, it must be stressed that our experimental design utilized cotreatment conditions rather than the pretreatment protocols used previously. This may partially account for these differences.

The mechanism by which IFN- γ can potentiate LPS stimulation is unknown. The existence of IFN- γ receptors on the surface of the monocyte has been demonstrated (6,13) and studies by Hamilton et al. (21) and Somers et al. (64) have shown increased levels of intracellular Ca^{++} and protein kinase C activity in murine monocytes following treatment with interferon. These findings have recently been confirmed in experiments by Celada and Schreiber (7). This evidence suggests that IFN- γ acts on human monocytes through a receptor mediated event which can elicit intracellular changes consistent with cell activation. However, the selective potentiation of *Bacteroides* over *Salmonella* LPS by IFN- γ cannot be explained by the mechanistic effects of interferon described to date. Further complicating the issue is the lack of evidence concerning the exact mechanisms by which LPS effects monocyte function. Clearly, more research is needed in these areas.

According to our results, we propose the following role for LPS in activating monocytes in the established adult periodontitis lesion or any lesion were chronic inflammatory and bacterial components are found. Once recruited, mononuclear phagocytes are stimulated by LPS released from the subgingival microflora, comprised in large part of *Bacteroides* species. This results in the release of PGE_2 and other soluble factors such as interleukin 1. The LPS stimulated monocytes also stimulate the release of IFN- γ from T lymphocytes. IFN- γ can then act in conjunction with LPS resulting in even higher levels of PGE_2 and/or IL-1 release from macrophages. LPS from *Bacteroides* species are capable of a greater pathogenic potential for two reasons: 1) IFN- γ potentiates the effect of *Bacteroides* LPS on monocyte secretion of PGE_2 and 2) lower concentrations of *Bacteroides* LPS would be capable of eliciting release of PGE_2 comparable to a higher dose of LPS without interferon.

In summary, this study provides additional evidence supporting the relatively weak endotoxic activity of LPS extracted from *Bacteroides* species when compared to *Salmonella*. However, these results have also demonstrated the capacity of IFN- γ to potentiate *Bacteroides* LPS stimulated release of PGE₂. This suggests a potential mechanism by which an otherwise weak LPS can stimulate the release of significant levels of PGE₂ in disease sites where inflammatory and immune processes are activated, as with adult periodontitis.

CHAPTER 4

Altered LPS-responsiveness in periodontitis patients, as measured by
monocyte release of PGE₂ and IL-1 β .

ABSTRACT

Lipopolysaccharide-responsiveness in human subjects was assessed through the examination of LPS-stimulated PGE₂ and IL-1 β release from counterflow isolated monocytes from patients with varying levels of periodontal destruction. This was performed in order to investigate a possible relationship between LPS-mediated secretory responses in monocytes and susceptibility to periodontal destruction in humans. Subjects were chosen based on apparent resistance or susceptibility to disease as measured by little or no periodontal destruction versus generalized severe destruction, respectively. Because IFN- γ can influence LPS stimulated responses, the effect of IFN- γ on the LPS stimulated release of PGE₂ and IL-1 β was also assessed. Peripheral blood monocytes were separated by counterflow centrifugation and cultured (10⁶/ml/well) with control medium or medium containing LPS from *Bacteroides gingivalis*, *B. intermedius*, *Actinobacillus actinomycetem-comitans*, or *Salmonella typhimurium*, with or without 10 Units/ml recombinant IFN- γ . Media were exchanged at 24 and 48 hours and culture supernatants assayed for both PGE₂ and IL-1 β by RIA. Patients classified as Susceptible to periodontitis, demonstrated 2-3 fold greater PGE₂ release than Resistant patients. This difference was observed with all LPS preparations over both the 0-24 hour and 24-48 hour culture periods. IL-1 β release, however, was not significantly different between patient groups. IFN- γ did not affect the LPS stimulated release of PGE₂ but significantly enhanced the release of IL-1 β . The IFN- γ effects were similar for both patient groups. These findings indicate that LPS-stimulated PGE₂ release from peripheral blood monocytes may correlate with susceptibility to periodontitis in human subjects.

INTRODUCTION

Research conducted with C3H murine strains has determined a genetic basis for differing host responses to bacterial lipopolysaccharide (LPS) (66). In these studies, *in vivo* tests of LPS lethality have been used to describe LPS susceptible (C3H/HeJ) and resistant (C3H/HeN) strains (74). Furthermore, lethality has been correlated with the capacity of LPS to elicit PGE₂ release from peritoneal macrophages isolated from the same animals (74). Additional work has established that in these animals the inherent susceptibility or resistance to LPS is related to the expression of a specific LPS response gene, located on chromosome 4 (75). Furthermore, immunological manifestations in the expression of this genetic information are not limited to monocyte function, as B lymphocyte blastogenesis can also be correlated with LPS susceptibility in these murine strains.

Existence of a variable susceptibility to LPS in humans has not been investigated to date, yet humans are afflicted with many diseases associated with bacterial LPS. This includes diseases such as chronic adult periodontitis which can lead to irreversible destruction of host tissues. Periodontitis is associated with a predominantly gram negative bacterial flora located in the periodontal pocket (32). LPS is a constituent of gram negative bacterial cell walls and has been found in greatest quantity in the subgingival plaque layer adjacent to the gingival tissues (14). Additional evidence has shown LPS to penetrate intact sulcular epithelium (59), result in vascular leakage (51), and correlate positively with clinical and histological signs of gingival inflammation (61,62). Furthermore, LPS preparations isolated from many suspected periodontal pathogens can elicit host responses including the localized Schwartzman reaction (36,69), limulus lysate gelation, chick embryo lethality (36), and bone resorption (24), as well as, specific cellular responses

including the release of PGE₂ (29) and IL-1 (2). Although there is a significant body of evidence implicating LPS as a pathogenic substance in periodontitis, little evidence exists demonstrating relative susceptibilities of human subjects to such LPS preparations.

That human subjects express variable susceptibilities to periodontal disease has been strongly suggested through longitudinal clinical studies of tea plantation workers in Sri Lanka (33). According to the results of these studies, a wide range of disease progression rates were observed which did not correlate with levels of bacterial plaque accumulated on tooth surfaces. Although variation in the composition of the bacterial flora undoubtedly contributes to disease progression, it would be highly speculative to attribute differences in progression solely to the variability in bacterial flora. Alternatively, the variation in susceptibility to periodontitis could be partially related to immune responses which are genetically determined. Little research has been conducted to define such a process in humans, however, the characteristics of adult periodontitis lends itself to studies concerning LPS-responsiveness of human cells.

Therefore, the present study sought to assess the LPS-responsiveness of subjects classified as Susceptible or Resistant to periodontitis. LPS stimulated release of PGE₂ and IL-1 β from peripheral blood monocytes served as the measure of LPS-responsiveness. Furthermore, because gamma interferon (IFN- γ) has been shown to potentiate these LPS responses in humans (2,42) and correct some aspects of the defective LPS response in C3H/HeJ mice (73), the capacity of IFN- γ to modulate PGE₂ and IL-1 β release was also examined in these patients.

METHODS AND MATERIALS

Patient Selection : Patients in good general health (free of any systemic diseases), with a minimum of 20 teeth, were asked to participate in the study if they fulfilled the selection criteria as depicted in Table 1. Seven Susceptible and six Resistant patients were identified. All patients were free of non-steroidal anti-inflammatory drug use for at least seven days prior to blood donation. A bleeding on probing measurement was performed immediately after blood drawing. This was used to assess whether a relationship could be observed between periodontal inflammation and LPS-induced monocyte secretory responses.

TABLE 1. PATIENT SELECTION CRITERIA

| CRITERIA | SUSCEPTIBLE | RESISTANT |
|---------------------------------|-------------|-------------------------------|
| Age | 30-60 yrs | 30-60 yrs |
| Mean Interproximal Pocket Depth | > 5 mm | < 3.5 mm no pockets > 5 mm |
| Radiographic Bone Loss | ≥ 40% | ≤ 10% |
| Plaque Index | >1 | >1 |

Cell Separation : Monocytes were separated from citrated whole venous blood by ficoll-Hypaque sedimentation (400 x g x 35 min.) at 10°C. The mononuclear leukocyte rich fractions were collected, diluted and washed two times in Ca⁺⁺ and Mg⁺⁺ free, phosphate buffered saline (PBS) (125 x g x 15 min.) at 10°C. The cells were then counted electronically (Coulter Electronics) and monocyte enrichment achieved by counterflow centrifugation (Beckman Instruments). Autologous plasma was added (final concentration of 1%) and monocytes sedimented (125 x g x 15 min.) and resuspended in MCDB 104 (37)

medium supplemented with 1% defined heat inactivated fetal calf serum (Hyclone), 100 U/ml penicillin and 100 mcg/ml streptomycin. Cells were counted and plated in plastic (Falcon Plastics) 16mm wells (1×10^6 cells/ml per well). At 2 hours the cells were washed two times in medium to remove any non-adherent cells. Cultures were incubated at 37°C in an H₂O saturated 5% CO₂ atmosphere. Cell viability was routinely confirmed by trypan blue exclusion. Treatments consisted of the following: LPS (10 ug/ml) or control medium, with and without IFN- γ (10 Units/ml). Media were exchanged at 24 and 48 hours and the supernatants assayed for PGE₂ and IL-1 β content.

LPS and IFN- γ Preparations : LPS was obtained from the following bacterial strains: *Salmonella typhimurium* (Difco Laboratories), *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* (kindly provided by Dr. Stan Holt, University of Texas Health Center at San Antonio). All LPS preparations were extracted by the hot phenol-water method of Westphal and Jann (76). *Salmonella*, *B. intermedius*, and *B. gingivalis* LPS preparations were found to contain 5.1 %, 6.9%, 11.9% protein, respectively, as measured by Lowry assay (35). Protein was undetectable in the *A. a.* LPS preparation. Recombinant IFN- γ was purchased from AMGEN Biochemicals with a specific activity of 1.6×10^7 Units/mg protein calibrated against NIH IFN- γ standard GG-23-901-530.

Assay for PGE₂ : Levels of PGE₂ in the culture supernatants were assayed by radioimmunoassay using absorption to dextran-coated charcoal. Anti-PGE₂ antibody and ³H-PGE₂ were purchased from Seragen and New England Nuclear, respectively. The anti-PGE₂ antibody demonstrates 100% cross-reactivity with PGE₁, however, other investigations in this laboratory have demonstrated that the E series prostaglandin released from human counterflow isolated monocytes consists of >97% PGE₂ when assayed by gas

chromatography-mass spectrometry (manuscript submitted). The PGE₂ levels in culture supernatants were extrapolated from PGE₂ standard binding utilizing logit-log transformation.

Assay for Interleukin 1 β : Levels of IL-1 β in the culture supernatants were assayed by radioimmunoassay purchased from Cistron. According to recent evidence, IL-1 β represents approximately 90% of the total IL-1 released from monocytes (46), with IL-1 α being 10%. The anti-IL-1 β antibody does not cross-react with IL-1 α , IL-2, tumor necrosis factor, or IFN- γ (Cistron technical bulletin). The IL-1 β levels in culture supernatants were extrapolated from IL-1 β standard binding utilizing logit-log transformation.

Data Analysis: Data are depicted in Tables 2-5 as ng/ml of PGE₂ or IL-1 β in culture supernatants. Overall differences between Resistant and Susceptible patients and interactions between variables were tested by three-way ANOVA using STATUS (Resistant or Susceptible), LPS(*A.a.*, *B. intermedius*, *B. gingivalis*, *Salmonella* and none) and IFN (yes or no) as independent grouping-variables and PGE₂ or IL-1 β as dependent variables. Differences between Susceptible and Resistant patients for each individual treatment were tested by one-way ANOVA as noted in Table legends.

RESULTS

Patient data: The average age for patients in the Susceptible and Resistant groups was 42.3 and 37.3 respectively. The mean interproximal pocket depth was 5.1 and 2.7 mm for Susceptible and Resistant patients, respectively.

LPS stimulated PGE₂ release: Tables 2 and 3 demonstrate the data for PGE₂ release during the 0-24 and 24-48 hour intervals. During the first 24 hour interval, the difference between Resistant and Susceptible patients in

LPS stimulation of PGE₂ release was statistically significant at the $p < .001$ level, when analyzed by 3-way ANOVA. LPS also resulted in significant effects at the $p < .001$ level. However, the effect of IFN- γ was not significant. Furthermore, no interaction between variables was detected by this analysis. One-way ANOVA was used to analyze differences between patient groups for each individual LPS preparation, with or without IFN- γ . As can be seen in Table 2, the differences between Resistant and Susceptible were significant ($p < .05$) with *Salmonella* and *B. intermedius* LPS (without IFN- γ), and *Salmonella*, *A. a.*, and *B. intermedius* (with IFN- γ). In general, monocytes from the Susceptible patients released approximately 2-fold greater PGE₂ with all LPS preparations tested.

During the 24-48 hour interval, the overall amounts of PGE₂ released were less than that observed during the initial 24 hour interval. However, when examining the difference between Susceptible and Resistant patients, a situation similar to the 0-24 hour interval was observed. As shown in Table 3, Susceptible patients released significantly ($p < .001$, by 3-way ANOVA) greater PGE₂ than Resistant patients. Using one-way ANOVA, significant ($p < .05$) differences were noted for *Salmonella* LPS without IFN- γ and *B. intermedius* with IFN- γ . Susceptible and Resistant patients differed in PGE₂ release by 3-fold during the second time interval. Again, interferon had no significant effect as assessed by 3-way ANOVA.

LPS stimulated IL-1 β release: Data in Tables 4 and 5 represent IL-1 β release from monocytes of Resistant and Susceptible patients during the first (0-24 hour) and second (24-48 hour) time intervals. During the 0-24 hour interval, the levels of IL-1 β were observed to be greater than the amounts of PGE₂ (Table 2) in the same monocyte culture media samples. More

TABLE 2. PGE₂ RELEASE DURING 0 -24 HOUR INTERVAL

| INTERFERON: | NO | | | | | |
|-------------|------|-------------------|-----------------------|-----------------------|----------------------|------------|
| | LPS: | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedius</i> | <i>B. gingivalis</i> | None |
| RESISTANT | | 3.32* ± .38 | 2.35 ± .35 | 1.65* ± .27 | 1.57 ± .35 | .23 ± .17 |
| SUSCEPTIBLE | | 7.58 ± 1.49 | 4.76 ± 1.17 | 4.65 ± 1.16 | 3.29 ± 1.52 | 1.13 ± .70 |

| INTERFERON: | YES | | | | | |
|-------------|------|-------------------|-----------------------|-----------------------|----------------------|------------|
| | LPS: | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedius</i> | <i>B. gingivalis</i> | None |
| RESISTANT | | 3.27* ± .34 | 2.07* ± .35 | 1.82* ± .35 | 1.56 ± .30 | .39 ± .25 |
| SUSCEPTIBLE | | 7.21 ± 1.19 | 5.08 ± 1.21 | 4.85 ± 1.05 | 3.43 ± 1.32 | 1.30 ± .55 |

Monocytes were isolated from 7 Susceptible and 6 Resistant patients. 10⁶ cells were cultured with the indicated LPS preparations (10 ug/ml) or control medium, with or without IFN- γ (10 U/ml). PGE₂ (ng/ml) was measured by RIA and depicted as the mean \pm SEM.

By 3-way ANOVA, significant differences were shown between:

- 1) Susceptible and Resistant patients, $p < .001$
- 2) Between LPS treatments, $p < .001$

*By 1-way ANOVA, significant differences were noted between Resistant and Susceptible patients for each individual LPS treatment, $p < .05$

TABLE 3. PGE₂ RELEASE DURING 24-48 HOUR INTERVAL

| INTERFERON: | NO | | | | | |
|-------------|------|-------------------|-----------------------|-----------------------|----------------------|-----------|
| | LPS: | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedium</i> | <i>B. gingivalis</i> | None |
| RESISTANT | | 1.36* ± .41 | .87 ± .11 | .46 ± .10 | .67 ± .21 | .14 ± .08 |
| SUSCEPTIBLE | | 5.75 ± 1.49 | 2.43 ± .78 | 2.07 ± .70 | 1.45 ± .81 | .53 ± .44 |

| INTERFERON: | YES | | | | |
|-------------|-------------------|-----------------------|-----------------------|-----------------------|-----------|
| LPS: | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedium</i> | <i>B. gingivialis</i> | None |
| RESISTANT | 2.00 ± .52 | 1.42 ± .34 | .80* ± .20 | 1.22 ± .40 | .18 ± .12 |
| SUSCEPTIBLE | 5.80 ± 1.60 | 3.03 ± .70 | 2.33 ± .61 | 2.13 ± .83 | .53 ± .20 |

Monocytes were isolated from 7 Susceptible and 6 Resistant patients. 10⁶ cells were cultured with the indicated LPS preparations (10 ug/ml) or control medium, with or without IFN- γ (10 U/ml). PGE₂ (ng/ml) was measured by RIA and depicted as the mean \pm SEM.

By 3-way ANOVA, significant differences were shown between:

- 1) Susceptible and Resistant patients, $p < .001$
- 2) Between LPS treatments, $p < .001$

*By 1-way ANOVA, significant differences were noted between Resistant and Susceptible patients for each individual LPS treatment, $p < .05$

TABLE 4. IL-1 β RELEASE DURING 0-24 HOUR INTERVAL

| INTERFERON: | NO | | | |
|-------------|-------------------|-----------------------|-----------------------|----------------------|
| | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedium</i> | <i>B. gingivalis</i> |
| RESISTANT | 27.65 \pm 3.35 | 15.63 \pm 3.57 | 11.39 \pm 2.26 | 15.18 \pm 5.23 |
| SUSCEPTIBLE | 25.69 \pm 4.30 | 14.25 \pm 3.67 | 13.31 \pm 3.63 | 11.11 \pm 4.02 |
| | | | | 1.45 \pm .82 |
| | | | | 3.58 \pm 2.83 |

| INTERFERON: | YES | | | |
|-------------|-------------------|-----------------------|-----------------------|----------------------|
| | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedium</i> | <i>B. gingivalis</i> |
| RESISTANT | 27.27 \pm 2.50 | 19.96 \pm 4.13 | 18.18 \pm 4.75 | 17.60 \pm 4.48 |
| SUSCEPTIBLE | 28.55 \pm 4.77 | 18.74 \pm 1.70 | 18.76 \pm 3.89 | 18.48 \pm 2.78 |
| | | | | 3.03 \pm 2.00 |
| | | | | 4.36 \pm 3.12 |

Monocytes were isolated from 7 Susceptible and 6 Resistant patients. 10⁶ cells were cultured with the indicated LPS preparations (10 ug/ml) or control medium, with or without IFN- γ (10 U/ml). IL-1 β (ng/ml) was measured by RIA and depicted as the mean \pm SEM.

By 3-way ANOVA, significant differences were shown between:

- 1) IFN- γ treatment, $p < .05$
- 2) Between LPS treatments, $p < .001$

TABLE 5. IL-1 β RELEASE DURING 24-48 HOUR INTERVAL

| INTERFERON: | NO | | | | |
|-------------|-------------------|-----------------------|-----------------------|----------------------|-----------|
| | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedius</i> | <i>B. gingivalis</i> | None |
| LPS: | | | | | |
| RESISTANT | 2.47 ± .37 | 1.31 ± .20 | 1.00 ± .20 | .98 ± .22 | .38 ± .08 |
| SUSCEPTIBLE | 1.96 ± .42 | 1.23 ± .20 | 1.07 ± .23 | .93 ± .19 | .42 ± .07 |

| INTERFERON: | YES | | | | |
|-------------|-------------------|-----------------------|-----------------------|----------------------|-----------|
| LPS: | <i>Salmonella</i> | <i>Actinobacillus</i> | <i>B. intermedium</i> | <i>B. gingivalis</i> | None |
| RESISTANT | 3.22 ± .59 | 1.74 ± .25 | 1.28 ± .22 | 1.59 ± .25 | .60 ± .16 |
| SUSCEPTIBLE | 2.87 ± .40 | 1.44 ± .22 | 1.45 ± .22 | 1.18 ± .26 | .54 ± .04 |

Monocytes were isolated from 7 Susceptible and 6 Resistant patients. 10⁶ cells were cultured with the indicated LPS preparations (10 ug/ml) or control medium, with or without IFN- γ (10 U/ml). IL-1 β (ng/ml) was measured by RIA and depicted as the mean \pm SEM.

By 3-way ANOVA, significant differences were shown between:

- 1) IFN- γ treatment, $p < .01$
- 2) Between LPS treatments, $p < .001$

importantly, however, no significant difference was observed between patient groups with respect to LPS stimulated release of IL-1 β . The only significant effect found by 3-way ANOVA was that of IFN- γ ($p < .05$) and LPS ($p < .001$). Interferon treatment resulted in approximately 30% greater release of IL-1 β from monocytes stimulated with all LPS preparations except *Salmonella*.

IL-1 β release decreased approximately 10 fold during the 24-48 hour interval (Table 5). Again, interferon and LPS had significant effects both at the $p < .001$ level; while no significant difference was found between Susceptible and Resistant patients.

DISCUSSION

The present study has partially characterized certain LPS-elicited secretory responses for monocytes isolated from patients with widely disparate levels of periodontal destruction. Although PGE₂ and IL-1 β may be important mediators in the pathogenesis of periodontitis, the primary goal of this investigation was the examination of LPS-responsiveness of monocytes and the relationship of those responses to disease severity in patients. A RIA was used to quantify IL-1 β so that chemical levels of IL-1 β rather than biologic activity was measured. Most previous investigations have measured IL-1 activity via the T lymphocyte blastogenesis bioassay. However, recent evidence indicates that IL-1 activity is influenced by the presence of IL-1 inhibitor (1,15,31) which can be released from activated monocytes (1). The RIA, which uses polyclonal anti-IL-1 β , would be less likely affected by an inhibitor of biologic activity. To our knowledge, the only disadvantage with the IL-1 β RIA is that levels of IL-1 α are not quantified. However, it has been reported that IL-1 β accounts for greater than 90% of the IL-1 released from LPS treated monocytes (46).

The patient selection criteria were intended to delineate subjects representative of the extreme ends of the periodontitis spectrum while demonstrating similar levels of plaque accumulation. Although these patients were not representative of the average patient with adult periodontitis, it was believed that these patients would offer the greatest likelihood of expressing altered immune responses. Another requirement for patient selection was that they be at least 30 years of age. It was felt that this was the earliest age when differences in LPS-responsiveness would be expressed by generalized periodontal status. Patients within the Susceptible group were diagnosed as having generalized severe adult periodontitis, whereas, the Resistant group represented patients with mild to moderate gingivitis. According to our hypothesis, the LPS-elicited secretion of PGE₂ and IL-1 β should be an inherent response unaffected by the stage of periodontal treatment at blood donation. Therefore, patients in the Susceptible group were chosen during various stages of periodontal therapy ranging from recently diagnosed but not treated, to patients under maintenance care. As an additional control for the influence of localized periodontal inflammation on peripheral blood monocyte function, bleeding on probing measurements were carried out at the time of blood donation. Analyses of bleeding on probing showed no correlation with monocyte LPS-responses.

LPS-responsiveness was assessed using LPS preparations isolated from three suspected periodontal pathogens and *Salmonella typhimurium*. Because the structure and biologic activity of *Salmonella* LPS have been well characterized (16), this was chosen as a standard LPS preparation for comparative purposes. Furthermore, *Salmonella typhimurium* is not an oral inhabitant and therefore would elicit LPS responses which were unrelated to

previous exposure through periodontal diseases. We have previously characterized the relative potencies of the LPS preparations derived from the suspected periodontal pathogens *A. a.*, *B. intermedius*, and *B. gingivalis*. All were shown to be less potent than *Salmonella* (manuscript submitted). However, in a separate study, the addition of 10 Units/ml recombinant IFN- γ was shown to preferentially increase the LPS-response of *Bacteroides* LPS. Therefore, the observed differences between LPS responses of *Salmonella* and *Bacteroides* were greatly diminished by the addition of IFN- γ . In the present study, no significant differences in IFN- γ effects on LPS-stimulated monocyte responses were observed between Susceptible and Resistant patient groups, regardless of the LPS preparation used.

The results from this study indicate that certain LPS-stimulated responses in monocytes from patients classified as Susceptible differ from those classified as Resistant. A two to three-fold greater release of PGE₂ was observed in monocytes of Susceptible patients, when compared to Resistant. This was observed with all LPS preparations over both time intervals and was not corrected by IFN- γ treatment. In contrast, the levels of IL-1 β assayed in the same culture supernatants were virtually identical between patient groups. The fact that IL-1 β release did not differ significantly between patient groups does not necessarily conflict with the genetic basis for LPS responses, as observed in the C3H murine model. Recent experiments have demonstrated that LPS from *B. gingivalis* and *E. coli* can stimulate IL-1 release from LPS-hyporesponsive (C3H/HeJ) mice (23), although to a lesser extent than that found with normal C3H/HeN mice. Therefore, IL-1 β release may not be as sensitive a measure of LPS-responsiveness as the release of PGE₂. Furthermore, the variability in IL-1 β release between subjects (Table 4) is much higher than that found with PGE₂ release, thus further contributing to

the difficulty of identifying small real differences, should they exist. Additionally, the fact that PGE₂ and IL-1 β release were not similarly effected within the same patient group suggests that LPS stimulated release of PGE₂ and IL-1 β are regulated by independent processes.

The relative differences between LPS preparations in the stimulation of PGE₂ release were consistent with a previous study (manuscript submitted) which ranked LPS potencies as *Salmonella* > *A. a.* > *B. intermedius* \geq *B. gingivalis*. This same ranking of potencies was also observed for IL-1 β release in this study.

The present study also demonstrated that IFN- γ alone, had no significant effect on PGE₂ and IL-1 β release from monocytes. However, treatment of monocytes with interferon significantly enhanced LPS stimulation of IL-1 β but not PGE₂ release. This effect was equal for both patient groups. Previous studies, in this laboratory and by others, have demonstrated IFN- γ enhancement of IL-1 (2) and PGE₂ (42) release when administered prior to LPS stimulation. However, the ability of IFN- γ to enhance LPS responses with simultaneous administration of both agents has only recently been examined. Simultaneous, rather than sequential treatment conditions were thought to more accurately reflect conditions at sites of inflamed periodontal tissues. A study recently conducted in this laboratory (manuscript submitted) demonstrated enhanced PGE₂ release from monocytes treated simultaneously with IFN- γ and LPS from *Bacteroides* species but not *Salmonella*. Interestingly, in the current study, a similar process was observed with IL-1 β release. IFN- γ treatment resulted in approximately a 30% increase in IL-1 β release with all LPS preparations except that of *Salmonella*. PGE₂ release also appeared to be enhanced by IFN- γ treatment during the 24-48 hour interval, however, these differences were

not statistically significant. Throughout the duration of this study, the activity of IFN- γ was monitored by repeated experiments with the same donor and through experiments using IFN- γ pretreatment conditions on LPS-stimulated PGE₂ release. No appreciable loss of IFN- γ activity was observed. That IFN- γ did not significantly enhance PGE₂ release in the present study, may be partially explained by the unusual criteria used for patient selection, since previous studies have utilized cells isolated from random normal donors without regard to periodontal status.

It has recently been suggested that the release of PGE₂ and IL-1 are not independent events and are actually interrelated. In a recent paper by Kunkel et al. (28), IL-1 was shown to stimulate PGE₂ release, whereas, exogenous PGE₂ treatment resulted in decreased IL-1 release. Furthermore, inhibition of PGE₂ release by indomethacin resulted in elevated release of IL-1. Therefore, elevated PGE₂ levels should result in decreased IL-1 release. According to the results of this study, Susceptible patients released significantly greater (2-fold) levels of PGE₂ without any pronounced effect on IL-1 β release. The cells used in the present study were human peripheral blood monocytes as opposed to the peritoneal macrophages used by Kunkel et al., which may account for the discrepancy in findings. Another possibility is that the RIA used in this study measured specific levels of IL-1 β and not IL-1 biologic activity, as in previous reports.

Although the periodontal condition of a patient can be influenced by many factors, of primary importance is the amount and specific bacterial composition of plaque. The predisposition of an individual for periodontal breakdown can only be expressed the presence of bacterial plaque. The results of the present study suggest that in addition to bacterial factors, host factors can influence cellular responses to a significant extent. Within the context of

periodontitis susceptibility, the following considerations become important. An overall increase in gram negative organisms within subgingival plaque could offer a greater threat to periodontal health via increased levels of LPS. However, because the potency of LPS varies with the bacterial source, some plaques may pose a greater threat to health depending upon bacterial composition. Therefore, generalized and localized periodontal destruction could be influenced by the combined effects of the amount and specific bacterial composition of plaque as well as the host response to the LPS released by the plaque organisms. The 2 to 3-fold increased release of PGE₂ demonstrated from the Susceptible patients examined in this study could result in an overall increased inflammatory and depressed immune response locally, thereby increasing the risk for periodontal destruction.

In summary, the present study consists of a preliminary investigation of the LPS-responsiveness of patients classified as Susceptible or Resistant to periodontitis. LPS-responsiveness was measured by monocyte release of PGE₂ and IL-1 β . LPS stimulation of monocytes from patients classified as Susceptible released 2-3 fold greater PGE₂ than Resistant patients. In contrast, IL-1 β release was similar for both patient groups. Simultaneous treatment of monocytes with IFN- γ was shown to enhance the amount of IL-1 β released from LPS stimulated monocytes but not PGE₂. Therefore, the altered PGE₂ release between patient groups was not minimized by IFN- γ .

DISCUSSION

The objectives of this research project were three-fold: 1) determine the relative capacity of LPS preparations from various suspected periodontal pathogens to elicit PGE₂ and/or IL-1 β release from human monocytes, 2) characterize the effects of IFN- γ on these LPS responses, and 3) determine if the capacity of monocytes to respond to LPS is related to susceptibility to periodontal destruction in human subjects.

The present findings indicate that LPS from *B. intermedius*, *B. gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Wolinella recta* can stimulate the release of PGE₂ and IL-1 β from human monocytes. However, the relative potencies vary between species, with all periodontal species except *Wolinella* being less potent than *Salmonella typhimurium*. The relative weakness of LPS from suspected periodontal pathogens (*A.a.*, *B. intermedius*, and *B. gingivalis*) was consistent with other investigations (26,36,41). Of interest was the finding that *Wolinella* LPS potency was equal to or greater than *Salmonella* and did not diminish over time as did all other preparations. However, comparison of *Wolinella* LPS potency is limited since it was prepared by a method different from all other LPS preparations. Furthermore, little information exists with respect to its chemical composition. Thus, these studies indicated relative potencies of the LPS preparations as follows: *Wolinella* \geq *Salmonella* > *A. a.* > *B. intermedius* \geq *B. gingivalis*. This ranking was found to be consistent throughout all experiments with the exception of *Wolinella* which was not used in other experiments, due to a limited supply. Although IL-1 β release was not reported (due to the high cost of the RIA) for the comparative experiments (chapter 1), a limited analysis of dose responses with all LPS preparations,

except *Wolinella*, was performed. Results demonstrated the same relative potencies as were found with PGE₂ release. Furthermore, the IL-1 β results from the clinical study demonstrated similar relative potencies.

Differences in chemical composition between LPS preparations and their influence on LPS-stimulation of monocyte secretory responses were discussed in Chapters 1 and 3. Additionally, the importance of protein contaminants in monocyte activation processes were also considered, however, protein contaminants have not been tested for their ability to influence human monocyte secretory responses. Although it is important to minimize natural protein contaminants within LPS preparations, specific cellular responses *in vivo* are likely to involve LPS which contains protein contaminants. Therefore, more research is needed on the effects of protein contaminants.

When considering LPS pathogenic mechanisms relating to monocyte secretory function, the potentiating effects of IFN- γ are clearly important. Diseases which involve LPS-activation of monocytes in the presence of T lymphocytes, are likely to result in elevated release of IFN- γ . This idea stems from the finding that small percentages (10%) of monocytes in combination with T lymphocytes and LPS, result in elevated IFN- γ production (30). In addition, separate studies in this laboratory have found that media from LPS-treated monocytes can replace the need for intact monocytes in this process. However, LPS alone or in combination with IL-1 β could not stimulate IFN- γ release from T lymphocytes.

The effects of IFN- γ on LPS responses in monocytes were characterized for both pretreatment and simultaneous treatment conditions. Interestingly, the magnitude of IFN- γ effects was significantly influenced by treatment conditions. A much greater effect was observed when IFN- γ was used prior to

LPS stimulation than simultaneously with LPS. This was found for PGE₂ measured by both RIA and labeled metabolite release. It should be recalled that only *Salmonella* LPS was used for this portion of the study.

It was believed that simultaneous exposure of monocytes to IFN- γ and LPS was more likely to represent conditions as they would occur *in vivo*, particularly in chronic adult periodontitis. Therefore, a thorough examination of the effect of IFN- γ under simultaneous treatment conditions was undertaken using LPS preparations from *B. gingivalis*, *B. intermedius*, and *Salmonella*. *A. a.* was also tested but results are not presented here. Dose responses were performed for LPS preparations and IFN- γ . Of primary interest was the observation that IFN- γ treatment resulted in up to 3-fold greater PGE₂ release from monocytes treated with either *Bacteroides* LPS, while minimal enhancement of the *Salmonella* LPS response was observed. The significance of this finding was that an otherwise weak LPS preparation was able to stimulate PGE₂ release in amounts similar to that of *Salmonella*. This finding may be important with respect to the pathogenicity of LPS in certain periodontal diseases, since high numbers of *Bacteroides* species have been well associated with chronic adult periodontitis (12). Furthermore, the weak potency of *Bacteroides* LPS without the influence of IFN- γ could lead to the incorrect conclusion that LPS is primarily a weak virulence factor for *Bacteroides* species.

The effect of IFN- γ on LPS stimulated IL-1 β release was not investigated during the initial experiments because the IL-1 β RIA was unavailable. However, Arenzana-Siesdedos et al. (2) presented convincing evidence that preincubation with IFN- γ could not only enhance LPS-stimulated IL-1 release but prolong the duration of time in which monocytes could respond to LPS. This finding is consistent with the data presented here (chapter 2) concerning

PGE₂ release. Furthermore, results from the clinical study have shown a statistically significant enhancement of IL-1 β release with IFN- γ and LPS cotreatment. Therefore, IFN- γ appears to enhance both LPS-stimulated PGE₂ and IL-1 release from human monocytes under both simultaneous and pretreatment conditions.

The effect of IFN- γ was not equal for all LPS preparations examined. In fact, simultaneous treatment with *Salmonella* LPS and IFN- γ did not significantly effect PGE₂ release. With respect to IL-1 β release in the clinical study, IFN- γ also failed to effect *Salmonella* LPS while enhancing all other LPS preparations. However, it would be incorrect to conclude that *Salmonella* LPS responses are not modulated by IFN- γ . The IFN- γ dose response data presented in chapter 3 indicated that higher doses of IFN- γ can enhance *Salmonella* LPS-stimulated PGE₂ release. In addition, Arenzana-Siesdedos et al. (2) utilizing LPS from *Salmonella*, demonstrated that pretreatment with IFN- γ greatly enhanced *Salmonella* LPS-stimulated IL-1 release. In the experiments presented in chapter 3, data were not reported concerning IFN- γ cotreatment effects on *Salmonella* LPS-stimulated IL-1 β release. However, supernatants from the experiments which tested larger doses of IFN- γ with *Salmonella* LPS (10 ug/ml) have recently been assayed for IL-1 β . IFN- γ (100 and 1000 Units/ml) was found to markedly increase *Salmonella* LPS-stimulated IL-1 β release. The fact that *Salmonella* LPS was much more potent in the stimulation of PGE₂ and IL-1 β release probably accounts for the difficulty in observing a substantial effect of IFN- γ . As stated in chapter 3, monocytes may be primed for a maximal stimulatory capacity to LPS when exposed to IFN- γ , regardless of the LPS preparation. When all the data are considered, a conclusion can be reached that IFN- γ consistently acts to enhance the potency of LPS to stimulate PGE₂ and IL-1 β release from human

monocytes. This effect occurs under simultaneous and pretreatment conditions; however, the magnitude of the effect varies with the bacterial source of the LPS.

The examination of LPS-responsiveness in clinical patients, classified as Susceptible or Resistant, produced some of the most interesting findings from this research project. The dramatic elevation in LPS-stimulated PGE₂ release in the Susceptible group could have profound effects on the immune and inflammatory responses *in vivo*. Elevated PGE₂ levels could act to suppress the immune response, elevate the inflammatory response, and cause greater bone resorption.

The hypothesis that patients may differ in the ability to respond to LPS is attractive to periodontology for a number of reasons. In periodontitis, only a thin layer of stratified squamous epithelium separates large quantities of bacterial LPS from cells involved in the regulation of immune and inflammatory responses. Furthermore, the fact that LPS can readily pass through sulcular epithelium and that antibodies to various LPS preparations can be detected in the peripheral blood of periodontitis patients, suggests that cells found within the connective tissue adjacent to the sulcular epithelium are exposed to LPS. The degree to which gingival cells are exposed to LPS is, as yet, poorly characterized. However, topical application of LPS to tooth surfaces in periodontally healthy dogs is sufficient to elicit alterations in vascular permeability in the adjacent gingival tissues (51).

The following proposed model can partially account for the variable susceptibility observed with chronic periodontitis. The response to LPS is variable between individuals such that a LPS-response characteristic of a resistant individual would be associated with the development of gingivitis or mild periodontitis whereas a response consistent with a susceptible

individual would be more likely to develop severe periodontitis. The overall amount of LPS and the specific bacteria from which LPS is produced would also play a major role in the severity and localization of periodontal destruction. For example, a person classified as susceptible who practices meticulous oral hygiene except in certain areas would likely only have severe periodontal destruction at those sites which consistently retain plaque. Therefore, individuals of equal age with localized or generalized severe periodontitis would be expected to demonstrate equal LPS-responsiveness. The localization of periodontal breakdown would correlate best with plaque accumulation whereas LPS-responsiveness would correlate with the severity of destruction. Also, depending on the types and numbers of specific organisms within subgingival plaque, there could be a greater or lesser potential for destruction. Therefore, the pathogenic potential of a subgingival plaque will depend on the summed LPS challenge of all microorganisms present. Generalized and localized periodontal destruction could be influenced by the combined effects of the amount and specific bacterial composition of plaque and the type of response to the LPS released by the plaque organisms. This point is important because it demonstrates how the specific and non-specific plaque hypotheses are consistent with the proposed model.

In summary, the previously described findings demonstrate a relationship between LPS-mediated responses in monocytes and susceptibility to periodontal destruction in human subjects. Although monocytes may participate in the pathogenesis of periodontitis, the response to LPS demonstrated by these cells is proposed to reflect overall LPS-responsiveness. Indeed, it is likely that other cell types are involved in periodontal destruction. However, it is proposed that the monocyte response to LPS

reflects the potential for these other cells to be directly involved with LPS-initiated periodontal destruction. Future work should help to clarify these concepts.

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